Electron Microscope and Chemical Studies on the Internal Membranes of *B. licheniformis* 749C and *B. cereus* 569

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C O N T E N T S

SUMMARY.

Studies have been conducted on the internal membranes ("mesosomes") of B. licheniformis 749c and B. cereus 569 by electron-microscopy, electron-radio-autography, and chemical analysis of the bacterial phospholipids.

Mesosomes are a feature of all Gram-positive organisms so far examined, but many reports of their structure and chemical composition conflict, and there is little agreement on their function. Previous work in this laboratory has established that B. licheniformis 749c has only one mesosome per cell, the mesosome consists of a series of concentric sheets, and it probably divides during cell division (Highton, 1969); a model for its behaviour in exponential cells, which show considerable heterogeneity in length, has been presented (Highton, 1969).

It has now been shown (Sect. II) that in B. licheniformis 749c the mesosome may be present in the spores, and, whilst the mesosome behaviour may be different in the first generation of outgrowth, its subsequent behaviour in the first series of cell divisions accords well with the model put forward, the heterogeneity in length arising on spore outgrowth.

The generality that Gram-positive rods have only one mesosome was tested by examining B. cereus 569 under identical fixation and growth conditions (Sect. III).

B. cereus 569 was found to have more than one mesosome per cell, with variable structure. An analysis of mesosome number and structure under different conditions was performed.

Attention was then directed to spore outgrowth in B. cereus 569 with the object of specifically labelling developing mesosomes with radio-active tracers. Using metabolic inhibitors chloramphenicol and Actinomycin D, it was concluded that development of mesosomes during outgrowth required mRNA synthesis (Sect. IV). Whilst the original approach above was not of value, evidence was obtained from the electron microscope of Actinomycin D resistant membrane synthesis, possibly of mesosomal origin. This phenomenon was investigated in exponential cells (Sect. V) and coupled with electron-radioautography of cells labelled with tritiated glycerol during A.D. treatment; further evidence for A.D. resistant membrane synthesis was obtained. A supporting analysis by thin-layer chromatography of extracted lipid (Sect. VI) showed that the lipids were labelled during A.D. treatment, in contrast to the cessation of labelled amino-acid incorporation into protein. B. licheniformis 749c similarly treated with A.D. did not give any electron-microscope evidence of A.D. resistant membrane synthesis.

From these studies it is concluded that B. licheniformis 749c and B. cereus 569 may have different mesosome systems with different functions and controls, and conflicts arising from work published elsewhere regarding

structure and function of mesosomes may be ultimately resolved with the recognition of mesosome diversity.

SECTION I

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Part i) INTRODUCTION

A fundamental feature of cellular organisation is the presence of various forms of membranous structures. All animal cells have a similar architecture of a limiting cytoplasmic membrane from which is derived an extensive internal membrane system, the Endoplasmic Reticulum, on which most of the cell's protein synthesis occurs (Palade, 1964; Campbell, 1967). Chromosomes and nucleoli are contained within a nuclear membrane, and numerous small membranous organelles occur in the cytoplasmic sap, viz. the Mitochrondria, sites for oxidative phosphorylation and the TCA cycle (Mahler, 1966; Slater, 1966); and the Golgi apparatus, a site of lipid and glycolipid synthesis and involved in the production of Lysosomes, small vesicles containing highly active nucleases and proteases considered to be involved with intracellular digestion, secretion of extracellular enzymes, and trans-membrane transfers of small molecules (Brachet, 1961; De Duve, 1969). In higher plants, the cytoplasmic membrane is surrounded by a rigid cellulose wall, and additional membranous elements - chloroplasts - containing chlorophyll and accessory photosynthetic pigments occur in the cytoplasm. Both chloroplasts and mitochondria have their own characteristic DNA and RNA species, and appear to be capable of nucleus-independent protein synthesis and

replication (Smillie, 1969; Kirk, 1970; Van Bruggen, 1966; Taylor, 1970; Borst, 1969). Although quantitative variations occur, for example, small lymphocytes have very little endoplasmic reticulum whereas plasma-cells are packed with it (Humphrey, 1970) the general principle of localisation of particular biochemical systems to specific independent membranous organelles is unaffected by cell size or degree of differentiation, and continues down the evolutionary tree to the filamentous fungi and algae (both of which have mitochondria and the latter chloroplasts very similar to those of higher cells) and into the Protista (Euglena sp has fairly typical mitochondria and chloroplasts). This clear division of labour within the cell stops, however, at the unicellular algae and the bacteria, and although extensive intracellular membrane systems may still be present, a unique function or set of functions is often difficult to ascribe to them and their degree of autonomy uncertain. In Anacystis nidulans, for example (a blue-green unicellular alga), a system of membranous lamellae parallel to the cell surface and in continuity with the cytoplasmic membrane is present, and it is fairly clear that these lamellae are closely associated with the site(s) of photosynthesis and may initiate cell division (Allen, 1968); however, in the case of the photosynthetic bacterium Rhodospirillum molischianum, Gibbs (1965) was unable to correlate variations in bacteriochlorophyll with changes in the chromatophore

areas, in this case a series of stacked discs, unless the cytoplasmic membrane was also included in the survey. The existence of intracellular membranous organelles in the Eubacteria has only recently been recognised, due to improvements in electron microscope techniques, and in almost no case can a function be unambiguously ascribed to them.

The work presented described attempts to increase knowledge of the electron-microscope structure, and function of two bacterial internal membrane systems, one in Bacillus licheniformis 749C, and the other in B. cereus 569, and there follows brief summaries of relevant aspects of bacterial cell morphology and its growth and division. A short survey of recent work on bacterial membrane systems and membrane structure in general is finally included in this introduction.

Part ii) General Morphology of the Gram-Positive Cell

This study is concerned exclusively with Gram-positive rod-shaped organisms (Bacilli). The basis for Gram positivity is probably a permeability function of the cell wall (Salton, 1963; Wistreich, 1969) and correlates highly (but not exclusively (Brown, 1970)) with the cell wall being constructed from a limited variety of polymers, namely the muramic acids, teichoic and teichuronic acids (Salton, 1964). These are discussed below, and accordingly reference to the Gram-negative organism and Gram-positive cocci will be made only in so far as may be relevant to particular points.

Together with the Clostridia, the Bacilli are distinguished in the Eubacteriales by the production of heat-resistant spores, but are separable from the Clostridia in being aerobic (Wilson, 1966). Plate 1 shows a thin-section electron micrograph of a vegetative cell of B. licheniformis 749C fixed in osmium/cyanide (Highton, 1969) and post-stained with uranyl acetate and lead citrate. The appearance is typical of a sectioned Bacillus species cell. The wall, 600 Å thick, is separated from the plasma membrane (P.M.) by a small gap of 100 Å traversed by fine fibrils; this gap may be real and could correspond to the site of "membrane" teichoic acids which appear to be external to the P.M. but internal to the wall (Archibald, 1968). Finer structure may be seen in the wall, but this depends very much on the preparation, staining procedures and

organism; Nermut (1967) found significant changes in the thin-section appearances of the walls of Bacillus megaterium and Bacillus polymyxa depending on the post-stain used after glutaraldehyde fixation. Evidence of ordered substructure in walls at the macromolecular level is difficult to obtain in sections on account of the loss of resolution due to the specimen thickness, but examples have been obtained from certain bacilli by shadow casting and negative staining (Nermut and Murray, 1967; Ellar, 1968), and from optical filtering (Finch, 1967). A fairly extensive freeze-etching study has been undertaken by Holt and Ledbetter (1969) showing very clear examples of microstructure, e.g. arrays of 6-8A-diameter globules in Bacillus polymyxa fracture-faces. The plasma membrane (P.M.) is typically trilaminar, 80-100 Å across, and encloses the cytoplasm which contains ribosomes (R) and a fibrillar nuclear area. The identity of the nuclear area with DNA has been established (Hopwood, 1960). The mesosome is very obvious (m), and in this case consists of a series of concentric membranes, approximately 60 Å wide separated by gaps of about 100 Å enclosed in an invagination from the plasma membrane. Most bacilli are flagellated, but the flagellae are usually removed during fixation and embedding, and no trace can be seen either of the basal organelle in the plasma membrane (Abraham, 1965; Cohen-Bazire, 1968; De Pamphilis, 1971) nor of their exit through the wall.

Part iii) Wall Chemistry in relation to the Plasma Membrane

Comprehensive reviews are available on the detailed chemical structure and organisation of polymers in the walls of Gram-positive cells (Osborn, 1969; Campbell, 1969; Hughes, 1968; Ghuysen, 1968). The synthesis of the mucopeptide component, an amidated polyglycan linked by short peptide "cross-bridges", probably occurs on the plasma membrane for it involves various lipid intermediates: for example in S. aureus a C55 isoprenoid alcohol carrier for glucosaminyl-muramyl disaccharides and a membrane-bound lipid phosphokinase which requires phosphatidyl glycerol and cardiolipin as co-factors (Higashi, 1970a, b). Similar isoprenoid carriers appear a constant feature of peptidoglycan synthesising systems (Roberts, 1968). Additionally, most Gram-positive cell walls contain varying amounts of teichoic and teichuronic acids. Glycerol-type teichoic acids (1:3 or 1:2 phosphodiester-linked polyglycerols often 2- or terminally 1/3 substituted with sugars or amino acids) occur mainly intracellularly and in association with the membrane (Archibald, 1968), but may also exist in the wall. Co-valent linkage to the mucopeptide has been demonstrated for some; for example, B. subtilis 168 glycerol teichoic acid is linked via a terminal phosphate bond to poly N-acetyl hexosamine and thence by a peptide bond to the mucopeptide (Young, 1967). Ribitol-type teichoic acids (poly-ribitol phosphates)

are restricted to the walls; like the glycerol type, many are substituted with amino acids and sugars. Covalent linkage to mucopeptide has been described for certain ribitol teichoic acids, as in S. lactis I2 (Button, 1966) and S. pyogenes (Munoz, 1967) both of which are somewhat atypical; and for the 1:5 phosphodiester linked N-acetyl-glucosamine ribitol teichoic acid of S. aureus (Ghuysen, 1965). The third type of acidic polymer, teichuronic acids (poly-uronides), often occur with teichoic acid; for example, B. licheniformis 6346 walls consist of 40% peptidoglycan, 35% glycerol teichoic acid and 13-18% teichuronic acid, the last being a poly N-acetyl glucuronic N-acetyl galactosamine (Hughes, 1968). This teichuronic acid is probably linked via a C1 (reducing) terminal of N-acetyl galactosamine to a glucose or N-acetyl glucosamine phosphate on the mucopeptide (Hughes, 1970). The relative amounts of teichoic to teichuronic acids, however, are highly dependent on growth conditions; in particular, phosphate starvation results in large increases in teichuronic acids with corresponding reduction in teichoic acids (Ellwood, 1970). Magnesium starvation, or competition by high concentrations of other cations, increases the teichoic acid content, and the magnesium binding capacity of the walls is relatively increased (Ellwood, 1970; Meers and Ellis, 1970). This apparent relationship of the teichoic acids to magnesium binding has suggested that they may be involved in controlling

cation mobilities (Heptinstall, 1970). Evidence for a magnesium pump in B. cereus T has been presented (Schmidt, 1971), and in B. licheniformis ATCC 9945 the efficiency of a magnesium-dependent teichoic acid synthesising system was unaffected by magnesium concentration if the preparation (wall/membrane fraction) had been pre-incubated with magnesium (Hughes, 1971). The synthesis of all these polymers probably occurs via nucleotide intermediates, for example in B. licheniformis ATCC 9946 (Burger, 1966):

1. Glucosyl-x + CDP Glycerol ⟶ Glycerol-P-Glucosyl-x + CMP
2. Glycerol-P-Glucosyl-x + UDP Glucose ⟶ Glucose-Glycerol-P-Glucosyl-x + UMP (x is an unidentified acceptor)

Similarly, CDP-ribose and uridine-phosphate precursors are thought to be involved in ribitol and teichuronic acids respectively (Glaser, 1964; Hughes, and Tanner 1970), and, whilst acceptors are not yet defined, there is evidence that teichoic acid precursors may share, and compete for, the same lipid-phosphate carrier as mucopeptide precursors (Hughes, 1971) a and b

Part iv) Physical Aspects of Cell Growth, Division and Sporulation

Rod-shaped cells grow by extending lengthways; the cytoplasmic volume with its contents (ribosomes and proteins), and the surface areas of the wall and membrane are increased, and it is usually coupled with DNA replication followed by cell division. Growth rate is largely determined by temperature and availability of nutrients, upon exhaustion of which cell death usually with lysis occurs (Dawes, 1965; Postgate, 1967). In Gram-positive rods, however, the terminal growth phase is usually succeeded by sporulation.

a) Wall, Membrane and Cytoplasmic Growth:Cytoplasm

The major macromolecular constituents of the cytoplasm synthesised during growth are proteins (structural and enzymic) and ribosomes. The mechanisms of protein synthesis and its regulation are broadly understood and accord well with the operon model of Jacob and Monod (1961). Most protein synthesis occurs on polysomes bound to membranes (Schaechter, 1963); in *B. licheniformis* 5244, 96% of all 70s ribosomes are membrane-associated, and 68% of these are polysomes (Van Dijk-Salkinoja, 1970). Additionally, the whole protein-synthesising apparatus is probably organised into a DNA-RNA-membrane complex, as has been partially isolated from *B. megaterium* (Tremblay, 1969), and from *B. subtilis* (Takeuchi, 1970), the latter as a nucleoprotein complex

consisting of DNA, RNA and protein in physical states suggestive of biological activity (nascent and free RNA and some RNA and DNA polymerase activity). These and theoretical considerations of molecular diffusion in relation to target sites for synthesis and/or incorporation make it highly likely that considerable internal organisation is present in the growing cell. Growth also requires an increase in ribosomes. In E. coli, new ribosomal subunits, as opposed to stable recycling ones (Schlessinger, 1969; Kaempfer, 1970), are synthesised from specific ribosomal proteins and RNA (Nomura, 1970) and probably assembled by a co-operative process (Mitzushima, 1970). Control of the stable RNA species, tRNA and rRNA, is probably transcriptional and is related to amino-acid supply. In E. coli an identifiable gene "RC" is involved, mutations at which produce the "relaxed" response on starvation of an auxotrophic amino-acid, namely a continuation of RNA synthesis (Edlin, 1968). Various explanations of this control have been offered, but it now appears likely to involve a DNA specificity factor ψ (psi) (Travers, 1970) similar to the σ (sigma) factor of Burgess (1969) responsible for the specificity of RNA polymerase for particular DNAs or regions of DNA, and requiring a guanosine tetraphosphate co-factor (Cashel, 1969). There is also evidence that rRNA is associated with membranes during, or shortly after, synthesis (Haywood, 1971). Investigation of a rifampicin-resistant mutant

of B. subtilis, rfr10 (Hussey, 1971), suggests that similar controls of ribosome synthesis to E. coli exist in Gram-positive cells, and the close linkage of the 5s and 23s rRNA cistrons in B. subtilis (Colli, 1971) suggests that at least the rRNA genes may be organised into an operon. Finally, the overall effect of ribosomal control mechanisms in B. licheniformis is to alter the number of sub-units synthesised at different growth rates, and not to change the individual biogenesis-time nor ribosome half-lives (Van Dijk-Salkinoja, 1971).

Wall and membrane: Intracellular growth is normally reflected by increases in the surface areas of the wall and plasma-membrane. The importance of functional organisation is readily apparent for wall growth where polymer chemistry and orientation specifies the permissible ways of expanding the structure (Weidel, 1964); however, as the wall is equivalent to an extra-membrane condensation, it is also clear that growth of a functional plasma membrane is a pre-requisite of balanced cell and wall expansion. Membrane growth exclusive of the internal membranes (part V) is considered first.

Balanced membrane synthesis involves the synthesis and assembly of structural proteins and lipids, and the topographically correct insertion of enzymes, whose functional dependence may rest with either the lipids or proteins: in E. coli, for example, vectorial

phosphorylation of sugars requires phosphatidyl glycerol (Milner, 1970) whereas in the same organism pleiotropic loss of nitrate reductase and formate dehydrogenase in chlorate-resistant mutants appears to be associated with loss of a structural membrane protein (Schnaitman, 1969; Onodera, 1970). The study of membrane growth is complicated by the possibility that the two distinct molecular species (protein and lipid) may have individual turnovers and insertion-points, and also change with growth phases and in different strains: for example, phosphatidyl glycerol and cardiolipin undergo rapid turnover in exponential cells of B. licheniformis 749 and 749C which decreases on entry into the stationary phase (Mormon, 1970). The relationship of lipid turnover or membrane growth to phenotypic expression appears to be variable; in an E. coli glycerol auxotroph (Fox, 1970) phospholipid synthesis ceases immediately on glycerol starvation, and there is a 10-fold decrease in the transport activity of the gal. system on subsequent induction, despite synthesis of galactosidase and "M" (transport protein (Scarborough, 1968)) up to 50% of normal. This suggests a requirement for new lipid synthesis for functional assembly. A similar glycerol auxotroph has been constructed from B. subtilis B24; during glycerol starvation some phospholipid turnover occurs but there is no difference in alkaline phosphatase inducibility. Induction of sucrase, however, is significantly affected (Mindich, 1970). Few studies have

been done on the control of membrane synthesis and stability of membrane structural protein. Chaloupka (1967) examined the turnover of ^{35}S-methionine in B. megaterium membranes and concluded that no extensive turnover occurred. However, there is evidence for a unidirectional control of the lipid composition by proteins. In S. aureus, a shift from anaerobic to aerobic conditions induces the formation of a membrane-bound electron transport system, concomitantly with which occur changes in the phospholipids (White, 1967) and rearrangements in their fatty acids (White, 1968). The unidirectionality of any control is illustrated in the glycerol auxotrophs mentioned above; both the Gram-positive and Gram-negative mutants continue to synthesise DNA, RNA and protein during glycerol starvation, and Mindich (1970) has clearly demonstrated that new proteins continue to be inserted into the B. subtilis membrane fraction. If the control of cytoplasmic synthesis can be regarded as a dilution function of regulator molecules, that of membrane synthesis may be more difficult, for, if the degree of specificity in the protein and lipid components is not critical to membrane assembly, as Mindich's study suggests, a permanent negative drain of possible regulatory precursors may exist. Control of membrane growth (Mindich's cells continue to grow slowly during glycerol starvation) may therefore rely on the physical assembly of enzyme systems whose terminal stage is membrane-associated, and involve

a control similar to that proposed by Fritz (1971) for L.D.H. where sub-unit interaction is a rate-limiting step limiting internal protein concentration. Conceivably, such a control could function through "mini-proteins" described for eucaryotic cell membranes (Laico, 1970). Few studies have established the topography of new plasma-membrane synthesis. Ryter (1967) employed potassium tellurite in an attempt to localise presumably membrane oxidation-reduction sites in thin-sections of B. subtilis; the distribution of reduced tellurite crystals suggested a central site of membrane synthesis, but tellurite is quite toxic and the result is not unambiguous. Morrison (1970) has made a comprehensive light-microscope autoradiography study on the distribution of grains from tritiated palmitic-acid-labelled B. megaterium cells. Palmitic acid turnover was shown to be very low, and an end-localised site of membrane growth seems a justifiable conclusion for this organism.

The requirements for wall growth are similar to those for membrane growth but with an added condition: membranes are complex crystalline structures which can theoretically increase by addition at any part of the array; cell walls, however, are basically co-valently bonded polymers and therefore growth points are physically and energetically restricted. It is generally accepted that the mucopeptide is the main rigid layer (Salton, 1964) and that the addition of new

material may require lytic enzymes to break down pre-existing wall to allow for insertion of new sub-units (Osborn, 1969; Ghuysen, 1968). There is some doubt, however, as to how cell shape is preserved; Schwarz (1971) has shown in E. coli that the conformation of the murein sacculus remains unchanged in different growth conditions despite significant chemical differences, and controls must therefore exist to limit possible modes of growth ~~to permissible ones~~. Such controls could involve the accessory polymers (e.g. teichoic acids) and wall-associated lytic enzymes as well as polymer structure and plasma-membrane organisation. Evidence for the functional significance of autolysins is two-fold: firstly, Shockman (1967), by measuring the release of ^{14}C lysine from labelled cells, demonstrated that the muramidase of S. faecalis (N-acetylglucosamine-N-acetyl muramic acid muramidase) was most active towards, and associated with, newly synthesised wall under conditions where old and new wall were equally susceptible to exogenous autolysin; secondly, Fan (1971) has isolated a mutant of B. subtilis with a temperature-sensitive autolysin whose growth at the restrictive temperature is markedly improved by the addition of normal autolysin or lysosyme. The very tight binding of autolysins to isolated wall preparations has suggested that these enzymes are "built into" the wall structure by co-valent attachment; there is now good evidence, however, that their association is

electrostatic (Pooley, 1970; Fan, 1970a). Furthermore, the S. faecalis autolysin exists in an inactive form which is activable by trypsin and much less tightly bound than the activated enzyme (Pooley, 1969). Latency in autolytic enzymes could provide a control for both growth and septation, and it is not clear whether the imbalanced wall synthesis, usually observable by wall thickening, that occurs for example in S. aureus during chloramphenicol treatment (Shockman, 1965), or in B. subtilis (Hughes, 1970) and S. faecalis (Pooley, 1969) during auxotrophic amino-acid starvation, is due to cessation of amidase synthesis or activation; autolysin was active in the B. subtilis thickened walls, but both active and latent autolysin was greatly reduced in the S. faecalis walls. Possibly related are the various isolations of "rod⁻" bacilli. Cole (1970) has described a nitrosoguanidine derivative of B. subtilis 168 which is temperature-sensitive for the rod shape; at the restrictive temperature, two of the three wall layers seen in sections are lost, and the mutation affects the amounts of both mucopeptide and teichoic acid in transformed recipients. Rogers (1970) examined several "rod" mutants of B. subtilis and B. licheniformis: two classes arose from B. subtilis, one (A type) reverting to rods in the presence of 0.8 M NaCl, the other (B type) stable. The salt in type A could be replaced by a restricted number of amino-acids, especially glutamine, and sections showed the spherical forms to have thickened

walls and disturbed septation. Possible explanations of this behaviour are salt-induced dissociations of autolysins and amidation functions of glutamine in respect to the cell wall; but independently of changing the degree of cross-linkage between wall polymers and the ability of lysins to correctly hydrolyse existing structure, gross morphological changes could be produced by altering the number and distribution of wall growth-points, about which there is little information for rod-shaped cells. For coccal cells, the original study of Cole (1962) on Streptococcus using fluorescine-labelled antibody suggested equatorial growth, a conclusion also reached from a thin-section study of the behaviour of lysosyme-resistant equatorial bands in S. faecalis by Higgins (1970). Briles (1970) used ^{3}H choline to show conservation of label at the ends and centres of chains of Pneumococcus, and hence equatorial growth. These studies, however, only reflect the growth modes of accessory polymers or individual molecules; Swanson (1969), for example, has demonstrated by ferritin-labelled antibody that the protein M antigen of various type A Streptococci is surface located, and commences regrowth from septal sites after removal by trypsin. Growth of accessory polymers may be independent of the mucopeptide. With respect to Gram-positive rods, Chung (1967) studied by light microscopy the distribution of grains from ^{3}H alanine (a mucopeptide amino-acid) in B. cereus pulse-labelled during chloramphenicol

treatment, and allowed to grow subsequently; several growth bands per cell were proposed although the sample examined appears to be very small, and no account taken of the possibility of mucopeptide turnover. Whilst in some species the mucopeptide is stable, as in B. megaterium (Pitel, 1970), in others there may be considerable lability; in B. subtilis W23 the mucopeptide half-life is only some 0.6 generation time independent of the growth rate, and the teichoic acid is also turned over (Mauck, 1970). An electron-microscope study by Highton (1971) on the regrowth of penicillin-resistant and other penicillinase mutants of B. licheniformis after penicillin treatment yielded strong circumstantial evidence that wall renewal occurred all over the cell during recovery by treatment with penicillinase. The use of cell perturbations to examine growth may, however, affect the wall growth system; for example, chloramphenicol in S. faecalis appears to alter the distribution of autolysin (Pooley, 1970), and penicillinase does not hydrolyse membrane-bound penicillin (Pollock, 1953). Support for an all-over mode of mucopeptide synthesis is indicated from the study of gelatin-induced reversion of B. subtilis protoplasts (Landman, 1968); the first sign of reversion is a dense line appearing round the cell, susceptible to lysosyme. Contrary to previous indications, it now seems clear that pre-existing wall (or wall fragments) is not an absolute requirement for new wall growth; besides

gelatin, growth on a membrane filter is sufficient to revert B. subtilis protoplasts (Clive, 1970) suggesting some kind of mechanical support is needed. Some confirmation for this comes from Cohen's (1971) study of stable L-forms of S. pyogenes; membrane fractions are able to form a polyrhamnose polymer once rhamnose is bound to the membrane although no mucopeptide is associated with it in L cells, as it is in normal S. pyogenes cells, despite the synthesis of mucopeptide precursors. This suggests that membrane attachment sites are the important factor.

In conclusion, it may be said that whilst certain aspects of cell growth are known in considerable detail, their inter-relationships cannot yet be understood; thus, the basis of an exponential and not linear mode of length increase between cell divisions demonstrated for some Gram-positive rods, e.g. B. cereus (Collins, 1962), must reflect rate increases in supply of precursors, and enzymes with short half-lives, correlated with the growth modes of wall and membrane.

b) Cell Division

Plate 2 shows a dividing cell of B. licheniformis. An incomplete septum divides the cell into two potential daughter cells, each with a nuclear area. A single mesosome lies between the septal edges, and in B. licheniformis and B. subtilis it will divide prior to completion of septation (Highton, 1970a, b). Division relationships

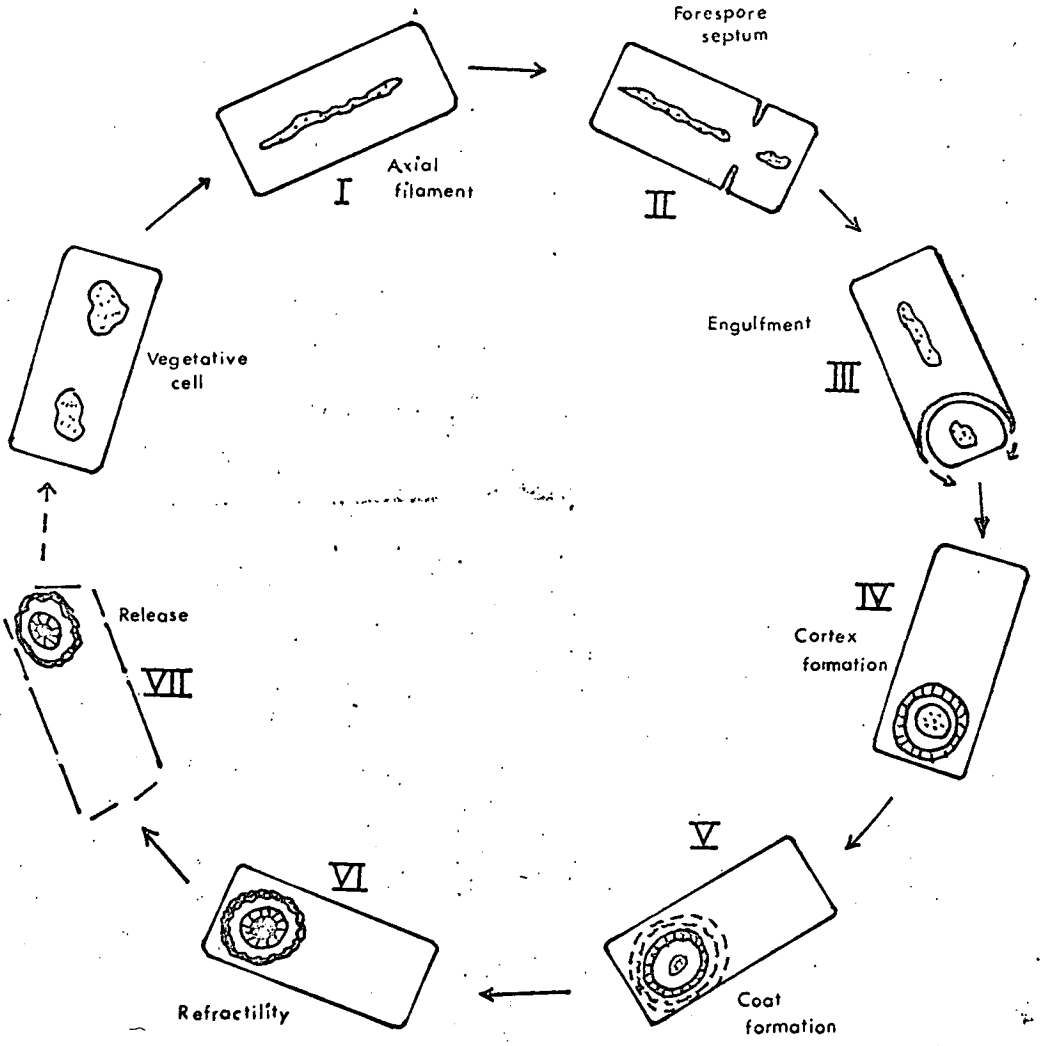
have been established for Gram-negative rods through studies on E. coli where division is normally seen in thin-section accompanied by cell waisting, construction of a septum nevertheless being involved (Conti, 1962). For E. coli, cell division is temporally related to the completion of a DNA replication round by a constant interval (Cooper, 1968) and hence to the number of replication rounds in progress as: i) in cells growing with a doubling-time of T minutes, chromosome replication is initiated every T minutes at a fixed point, and ii) the capacity for initiation is a result of T minutes uninterrupted protein synthesis, interruption delaying initiation by a time equal to the interruption (Helmsetter, 1968). It has been proposed that the DNA is physically separated by membrane growth (Jacob, 1963) and a consistent model for total cell growth based on a "unit-cell" hypothesis experimentally derived (Donachie, 1970). Some similarities have been found in Gram-positive rods; in B. subtilis, for example, the DNA is apparently membrane-associated (Snyder, 1969; Ivarie, 1970) but the regulation of chromosome replication may be different (Copeland, 1971). The first sign of septation is an indentation in the plasma membrane and a local convexity on the inner surface of the wall; centripetal extension of both eventually produces a complete septum, which later splits down the middle releasing the two cells. The long-recognised association of mesosomes with septa is not understood. The

prime mover of septation cannot be identified with either wall or membrane, although division of protoplasts (Kusaka, 1971) and mesosomes at least demonstrates wall-independent membrane division. In coccal cells, Higgins (1970) has shown that septation sites are initiated and then arrested during a period of peripheral wall growth. Septation then recommences and, after final closure, a rapid increase in thickness of the completed septa occurs. It is probable that septation in the Gram-positive rods is a different growth process from adjacent cylindrical growth, and may have some resemblance to coccal septation; Ellar (1967) found that septa of synchronised B. megaterium cells underwent a similar thickening phase, and whilst septa may appear planar before separation, the ends of some rods (e.g. B. cereus) are rounded. Thus, the ends must be remodelled either by additional synthesis or redistribution. Electron-microscope observations by Highton (1971) on the regrowth of B. cereus cells after penicillin treatment, and the discontinuous incorporation of DAP into synchronised B. subtilis cells coincident with division (Dadd, 1968) suggest that end-walls are differently made to cylindrical ones and conserved. Cell separation after septation is probably achieved by mucopeptide hydrolysis, as demonstrated by the dechaining effect of autolysin in B. subtilis (Fan, 1970b). Previc (1970) has proposed a geometrical model of cell division in rods and spheres, based partly on molecular diffusion

and chemical differences in the mucopeptide of rods and spheres at the third position of the peptide side-chain, and an attempt at a unifying model put forward by Higgins (1971). This model involves insertion of unidirectional wall elongation sites which are converted to bidirectional cross-wall sites, and a change in the orientation of parallel-stacked polymers. Whilst having elegant features, certain aspects are not considered; for example, mucopeptide polymers may be helical, not linear, and it is not necessary for right-angle bends to be made in them to achieve a right-angled septum. Considerations of the molecular distances involved suggest that the distortion produced without changing the chemical orientation of mucopeptide polymers would be very small (1 degree/disaccharide unit) and, perhaps more likely, the rounded end could be constructed geodesically (Highton, pers. comm.). Additionally, the different physical construction of walls and membranes mentioned previously strongly suggests that wall septation and membrane septation involve quite different modes of respective septal growth and initiation.

c) Sporulation

All aspects of bacterial sporulation have been recently extensively reviewed (Murrell, 1967; Gould, 1969). Briefly, sporulation follows derepression of the sporogenic genes in the chromosome. The normal state of repression may be associated with glucose



Stages in spore development, after KORNBERG, 1968.

(Ann. Rev. Biochem. 37, 51 1968)

catabolites and sporulation with nutrient lack; "sporogenic factors" may also be involved, and sporulation may therefore occur in continuous culture (Srinvasan, 1964). A number of morphological stages have been defined, redrawn in Figure 1. Essentially, a piece of DNA becomes segregated from the mother cell by the production of a membranous septum and is re-invested with a second layer of plasma membrane by regrowth of the mother-cell membrane around it; mesosomes are closely associated with this stage, and at least one appears to be enclosed in the future spore although their subsequent fate has not been studied. All subsequent stages concern the production of new layers round the protoplast and the maturation of the spore into a refractile heat and radiation resistant structure, capable of rapid germination into a vegetative cell. In these stages, secretion (presumably) of precursors between the two membranes produces a wide cortex composed of mucopeptide and calcium dipicolenate; the dipicolenate is closely associated with heat resistance and it has been suggested that the resistance of the mature spore to physical agents is due to dehydration, undoubtedly present, caused through a physical contraction of the cortex, in a way analagous to squeezing a wet sponge. The existence of compartmentalised and exchangeable water in the spore, however, suggests that other mechanisms such as hydrophobic exclusion may operate. The DNA segregated into the

fore-spore may consist of multiple genomes (Goldring, 1968) or in certain auxotrophs at least be semi-replicated (Ephrati-Elizur, 1971); its eventual resistance to various radiations implies a different physical state from vegetative DNA, perhaps the dehydrated "C" form. Following cortical development, spore coats and an exosporium are added around the cortex by condensation and beneath the mother cell wall respectively which then usually sloughs off. The various stages of development require sporulation specific enzymes, and cells become committed to the various stages at particular times (Sterlini, 1969); many sporogenic mutants are known whose morphological arrest can be correlated with biochemical deficiencies (Waites, 1970). The switch from vegetative to spore "genome" transcription appears to involve a sigma factor (Losick, 1969) and mutants altered in RNA polymerase may have disturbed morphology (Doi, 1970). Sporulation involves considerable changes in mucopeptide (Pitel, 1970), lipid (Lane, 1970) and ribosome (Hussey, 1971) metabolism as reflected for example in the occurrence of the -lactam of muramic acid (Warth, 1969) in the cortex and the enclosure of defective ribosomes in the spore (Idriss, 1969), and has been regarded as a modified cell division (Hitchins, 1969). Plate 3 shows a section of a mature spore of B. licheniformis. There is no information of detailed organisation within the mature spore protoplast which appears essentially

featureless as there appear to exist permeability barriers especially to osmium fixatives (Rode, 1962). Elegant demonstrations of surface structure in the various layers have, however, been obtained from freeze-etching and replica studies (Holt, 1969), which in some cases have been correlated with properties such as surface charge (Watanare, 1970) and the spore germination characteristics (Rode, 1968). A brief summary of germination may clarify aspects of the experimental work on germination presented later. In the dormant state, the spore has virtually no metabolic activity although various active enzymes may be present, for example alanine racemase. Additionally, the protoplast is devoid of stable mRNA and its protein synthesising capability defective (Idriss, 1969; Halvorson, 1966). Reversion to the vegetative cell proceeds in two stages: i) Germination; ii) Outgrowth. Germination is defined as the loss of phase-brightness and heat-resistance, and occurs very rapidly (one half to two minutes) in response to various external germinative agents ("triggers"); alanine and inosine are particularly effective for some spores, and specific initiation factors have also been described (Vary, 1968). During germination there is a rapid release firstly of muco-peptide (Dring, 1971), then calcium, dipicolenic acid and small quantities of peptides. In section, the ribosomes and often DNA become more visible, and most of the cortex disappears suggesting that it is the initial

"trigger" site (cortex-less mutants lyse before producing mature spores (Pearce, 1971)). Surface changes have also been found (Hashimoto, 1971). Apart from the ability to repair DNA (Terano, 1969) and an increase in respiratory activity, no macromolecular synthesis occurs. This requires exogenous nutrients, and, when supplied, outgrowth commences. A rapid increase in permeability and consequent swelling takes place, and if outgrowth is to occur, firstly RNA, protein, and, much later, DNA syntheses start. Outgrowth establishes the full capacity of the vegetative cell, and only after this does emergence of the cell from the spore remnant occur. Germinated spores are not necessarily committed to outgrowth, and even outgrown cells may revert to spores in the absence of sufficient nutrients (Mackechnie, 1970). Outgrowth is recognised microscopically by full visibility of DNA (which may be re-distributed (Rousseau, 1966)), ribosomes and plasma membrane. Mesosomes, however, appear to be absent until later, and the first two experimental sections examine mesosomal history during germination and outgrowth in two spore systems, B. licheniformis 749c and B. cereus 569. To complete this introduction, a short section on the current understanding of mesosomal morphology and function in vegetative cells follows.

Part v) Internal Membrane Systems of Bacteria

The present study is concerned solely with the mesosomes of two Gram-positive bacteria. Other systems of internal membranes exist, for example, those in the methane-oxidizing bacteria (Whittenbury, 1970) and photosynthetic bacteria; mesosomes are operationally defined as sacs of plasma membrane containing concentric lamellae or vesicles (or a combination) presumed to be membranous from their electron-microscope appearance, and are widely distributed in the Gram-positives. They also occur in the Gram-negatives but with a greater variability; those in Caulobacter sp. are prominent (Poindexter, 1964) whereas those in E. coli are very small except in certain mutants or under certain conditions (Pontefract, 1969, 1970). Despite considerable study since the original observation of "peripheral bodies" in B. cereus by Chapman (1953), there is no general agreement on their structure and function for a number of reasons. Firstly, a standard fixation process (Ryter, 1958) is a recent development, and the fine structure of mesosomes is highly dependent not only upon the fixative used (Burdett, 1970) but on the treatment the cells receive during fixation (Highton, 1969). Secondly, it seems clear that some organisms, e.g. B. licheniformis, have only one mesosome which divides during cell division (Highton, 1970a) whereas others, e.g. B. cereus, have several, none of which divide (this study). The probability, therefore, that

all mesosomes have the same function is reduced, and finally this function may change with growth phases or conditions and be reflected in structural alterations (Highton, 1971, unpublished). Suggestions for mesosome function have come from two approaches: i) structural analysis, and ii) biochemical dissection of cell fractions, and from both has come evidence for its involvement in variously wall or membrane growth, DNA replication, or respiration. A review is available (Ryter, 1969), and the approaches mentioned above can be summarised very briefly:

i) Structural analysis: The almost invariable association of a mesosome with cell septa (for example, Imaeda, 1963; Ellar, 1969; Highton, 1970) has suggested that they are involved with cell division or wall growth; their frequent association with nuclear material in section and during protoplasting (Ryter, 1964 and Landman) has allowed them to be used in DNA replication models, and also as a site for transformation (Wolstenholme, 1966); and the deposition of tellurite crystals on the mesosomal sac has provided evidence for partial involvement in respiration.

ii) Biochemical analysis: Support for respiratory functions has come mainly from the identification of cytochromes in membrane fractions (Ferrandes, 1966; Ghosh, 1969; Reaveley, 1969); all these studies employ a similar technique to separate mesosomal membranes. Studies by Ryter (1964) and Fitz-James (1964) showed

that during protoplasting by lysosyme, mesosomes were lost from the cell interior, and long tubular structures then appeared attached to the spherical protoplasts, presumed to be extruded mesosomes.

Consequently, mesosomal fractions are usually defined as membranes remaining after removal of the protoplasts by centrifugation. This assumption is open to the severe criticism that the relationship of such membrane fractions to *in vivo* mesosomes is most uncertain; for example, construction of a sphere from a rod-shaped envelope requires extensive surface reorganisation from which process membrane fractions could arise, and there does not seem to be any discussion on whether the number of tubule attachment points is related to the average number of cell mesosomes. That the "mesosome fractions" may be different from the protoplast membranes, for example in specific activity in lipid-labelling experiments (Ghosh, 1969), seems proven but until the derivation of the fractions is absolutely clear the significance must remain obscure.

A final point to consider is whether mesosomes are in any way essential to the cell. As yet, no mesosome mutants are available, but observations on the considerable delay in re-synthesising mesosomes during reversion of protoplasts (Landman, 1968) and their absence in growing and dividing protoplasts (Kusaka, 1971) suggests they are not essential for any of the functions so far proposed, although their presence in

the protoplast membrane cannot be excluded.

It is thus clear from the above and previous parts that much of the present confusion concerning the physical aspects of cell growth lies with an inability to describe the behaviour and function of mesosomes and the part they play in the topographical distribution of the various synthetic processes. The object, therefore, of the work presented is to clarify mesosomal behaviour in two organisms, and to develop a system whereby any mesosomal fraction isolated for biochemical analysis can be unambiguously related to the mesosome seen in section, using the technique of radio-active labelling and electron radio-autography.

SECTION II

Mesosomes during germination and outgrowth of spores of *B. licheniformis* 749c

SECTION II

Introduction

Exponential cells of B. licheniformis 749c have a single lamellar mesosome (Highton, 1969). A model for its behaviour was presented describing the relative growth of the cell past the mesosome which comes to lie centrally at the time of cell division and then divides, its division being physically separable from cell septation. This model was based on analysis of a large number of randomly chosen axial sections which showed a very high correlation of mesosome position with septation sites; sections showing mesosomes in a state strongly suggestive of division have also been observed (Highton, 1970a). However, the cell lengths in the analysis were distributed over a range of more than twice the shortest cell length, and the subsequent scatter in lengths and mesosome positions made it necessary to postulate a continuum of initial cell lengths in a heterogenous population. As the population was derived from spores, and nutrient auxotrophs are not readily available from B. licheniformis 749c from which to generate a synchronised culture, it was considered profitable to examine the mesosome distribution in germinating spores to establish firstly their position of origin and secondly whether the distribution in cells of the first division cycles after germination differed from that in fully established exponential

growth. It was shown that full mesosomal development occurred later, and possibly in random positions, during early outgrowth, and the subsequent distribution of mesosomes and cell lengths was similar to that found in a population of mid-exponential cells.

Materials and Methods

Organism: Spores of B. licheniformis 749c (penicillinase constitutive) were harvested by centrifugation from 48-hour cultures of 500 ml "S-S broth" (casamino acids 10 g/L, potato extract 10 g/L, yeast extract 2 g/L and Collins' Special Salts solution (P. Hill, M.Sc. Thesis, Edinburgh, 1969) 2 ml/L) incubated at 37°C aerobically. The preparations were cleaned by a series of at least six washes in distilled water (3,000 rpm for 10 min. per 20 ml suspension) followed by a discontinuous sucrose density-gradient centrifugation of 15, 30, 45 and 60% sucrose w/v, 10 ml each fraction, spun at ~~3,500 rpm~~ 2,500 g for 15 min. The 30% fraction was taken, the sucrose removed by a further series of three washes, and the clean spore suspension heat-treated at 65°C for 30 min. A final series of 2 washes in water followed. Spores were kept at 4°C in distilled water suspension.

Total counts were performed by phase microscopy using a Neubar counting chamber and verified on an electronic counter.

Viable counts were performed either by spreading known aliquots from ten-fold dilutions onto nutrient

agar, or by inoculating them into 5 ml 0.6% water-agar at 45° and immediately pouring onto a nutrient agar base. All counts were done in triplicate, and incubated at 37°C after drying.

Purity was checked on a casein-hydrolysate-salts agar containing 0.75% Polyvinyl alcohol, subsequently flooded with iodine and penicillin solutions to detect penicillinase production as described by Sherrat (Ph.D. Thesis, Edinburgh, 1969).

Germination: Germination was assessed on loss of both phase-brightness and malachite green retention (Mackay, 1965), and the germination rate for the population measured by the loss of heat resistance of distilled water suspensions to 65°C for 15 min. Two germination procedures were used: i) germination in L-broth (tryptone 10 g/L, NaCl 10 g/L, yeast-extract 5 g/L) in which outgrowth also commences, and ii) germination in the medium of Foerster (1966) consisting of L-alanine and inosine 0.ϕ01 M in 0.01 M sodium phosphate buffer pH 7.1. This medium is nutrient-deficient but contains specific germinants effective for a variety of Bacillus sp. spores. Spores thus germinated were subsequently transferred to the outgrowth medium of Sakikibara (1969). All experiments were conducted at an average spore concentration of 10^8/ml in flasks having a volume to culture ratio of 5:1, shaken at 37°C.

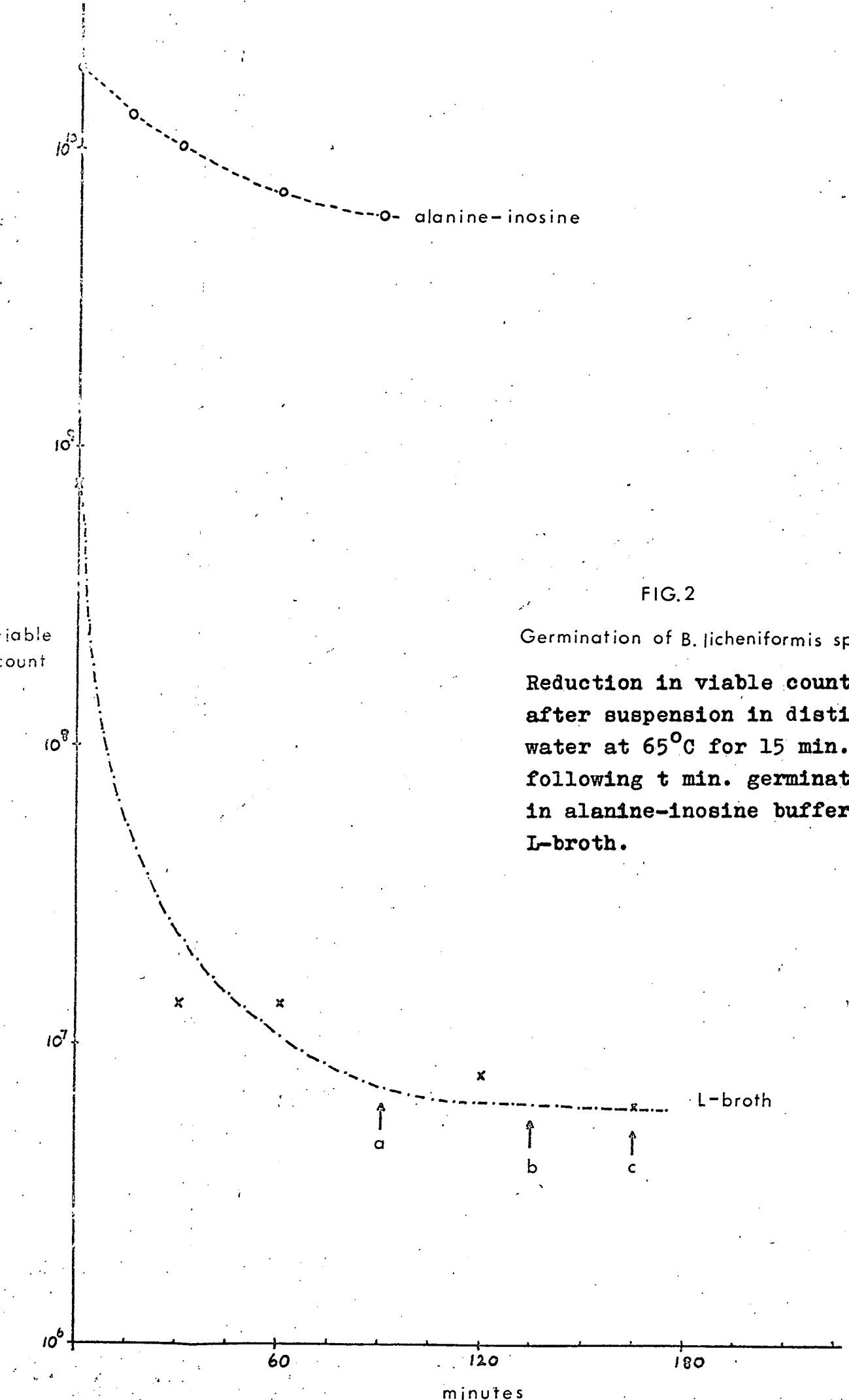


FIG. 2

Germination of B. licheniformis spores

Reduction in viable count after suspension in distilled water at 65°C for 15 min. following t min. germination in alanine-inosine buffer or L-broth.

Electron microscopy: Specimens for sectioning contained at least 10^8 particles. All specimens were pre-fixed and fixed in osmium/cyanide (Highton, 1969), dehydrated in graded acetone steps and embedded in araldite. Sections were cut on a Huxley microtome using a diamond knife, and post-stained with lead citrate. Grids were viewed in a Siemens Elmiskop 1A electron microscope operating at 80 KV, at magnifications of 10-20,000 times.

Results

The spores used were 99% viable (Fig. 1). Single colonies arising from the plating of appropriate dilutions were constitutive for penicillinase and had identical colonial morphology.

Figure 1. Viability of Sucrose Fractions.

Sucrose Fraction	Viable Counts	Total Counts		% Viability
		Coulter	Micro	
15%	2.5×10^8	1×10^{10}	8.5×10^9	10
30%	1.4×10^{10}	3×10^{10}	1.5×10^{10}	99
60%	2.6×10^9	Excess debris	1.6×10^9	60

Germination characteristics: These are presented in Figure 2.

a) Germination in alanine-inosine-phosphate buffer: Germination was poor in this medium, less than 90% germinating in one hour. Few spores progressed beyond

complete phase-darkening and swelling, although some outgrowth and a few free cells were seen after 8 hours. Subsequent transfer to outgrowth medium resulted in a long delay before exponential growth was established (Fig. 3).

Figure 3. Spores germinated in germination medium for 6 hrs. and transferred to stated media. Doubling times and cell growth measured by increase in optical density at 620 mu.

Medium	Doubling time mins.	Log-phase establishment
L-broth	33 mins.	6 hrs.
O.M.	70 mins.	10 hrs.

b) Germination in L-broth: Approximately 99% of the population had germinated in 30 min., and outgrowth proceeded. At 1½ hours (point a) about 10% were judged by phase microscopy to have the beginnings of an outgrowth tube, and at 2¼ hours (point b) most germinated spores were visibly outgrowing, few free cells being present. At 2¾ hours (point c) there was a significant number of free cells, and many outgrowing cells appeared to have septa.

Electron Microscopy

The resting spore: Dormant spores (Plate 3) are 0.5 - 0.8 u long and 0.3 - 0.5 u wide. In section,

the exosporium, approximately 60 mu thick, is composed of an outer amorphous layer (which may have surface fine structure, arrowed) and an internal lamellated layer, closely applied to the spore coats (c) which consist of a series of concentric lamellae each about 350 Å wide. Both the cortex and protoplast appear structureless, although occasionally a curved line of increased density is visible (Plate 4) which may be artifactual.

Membrane development during germination in alanine-inosine buffer: Specimens for electron microscopy were taken at 30 and 90 min. and eight hours. Although germination was far from synchronous, it was possible to construct a sequence of events from selected sections. The earliest sign of germination is the demarcation of the spore protoplast from the cortex (Plate 5); this demarcation becomes more evident, and a dense line appears around the protoplast in the region of the cortex which becomes reticulated (Plate 6). Subsequently, the plasma membrane becomes visible, and the amorphous appearance of the protoplast is lost (Plate 7). Internal membranes may be visible at this stage in some cells (Plate 8). Eventually, the protoplast assumes an appearance similar to that of a vegetative cell within the spore integuments, the DNA being fully visible, and the dense layer round the plasma-membrane contrasting with the almost complete disappearance of the cortex

(Plate 9). The occurrence of membranous structures (Plates 10 and 11) increases with the time the spores are left to germinate as shown in Figure 4; the relationship of the type of structures shown in Plate 12 to the obvious vesicular ones in Plates 10 and 11 is unclear, and therefore such cells were excluded.

Figure 4. Sections of spores germinated in germination medium for the stated times showing membranous structures. Selection: good overall preservation, minimum obliquity, and obvious structure (vesicles, lamellae).

Germination Time	With Membranes	Without Membranes
90 mins.	26 (26%)	74 (74%)
8 hours	77 (50%)	80 (50%)

An example of a lamellar membrane-like structure is shown in Plate 13. Prolonged incubation in germination medium allowed some vegetative outgrowth to occur (Plate 14); this was very infrequent, and produced highly abnormal cells with thickened and distorted walls, and reduced ribosome content, although membranes resembling mesosomes were sometimes present. Nevertheless, it would appear from Plate 14 that some form of cell division could occur.

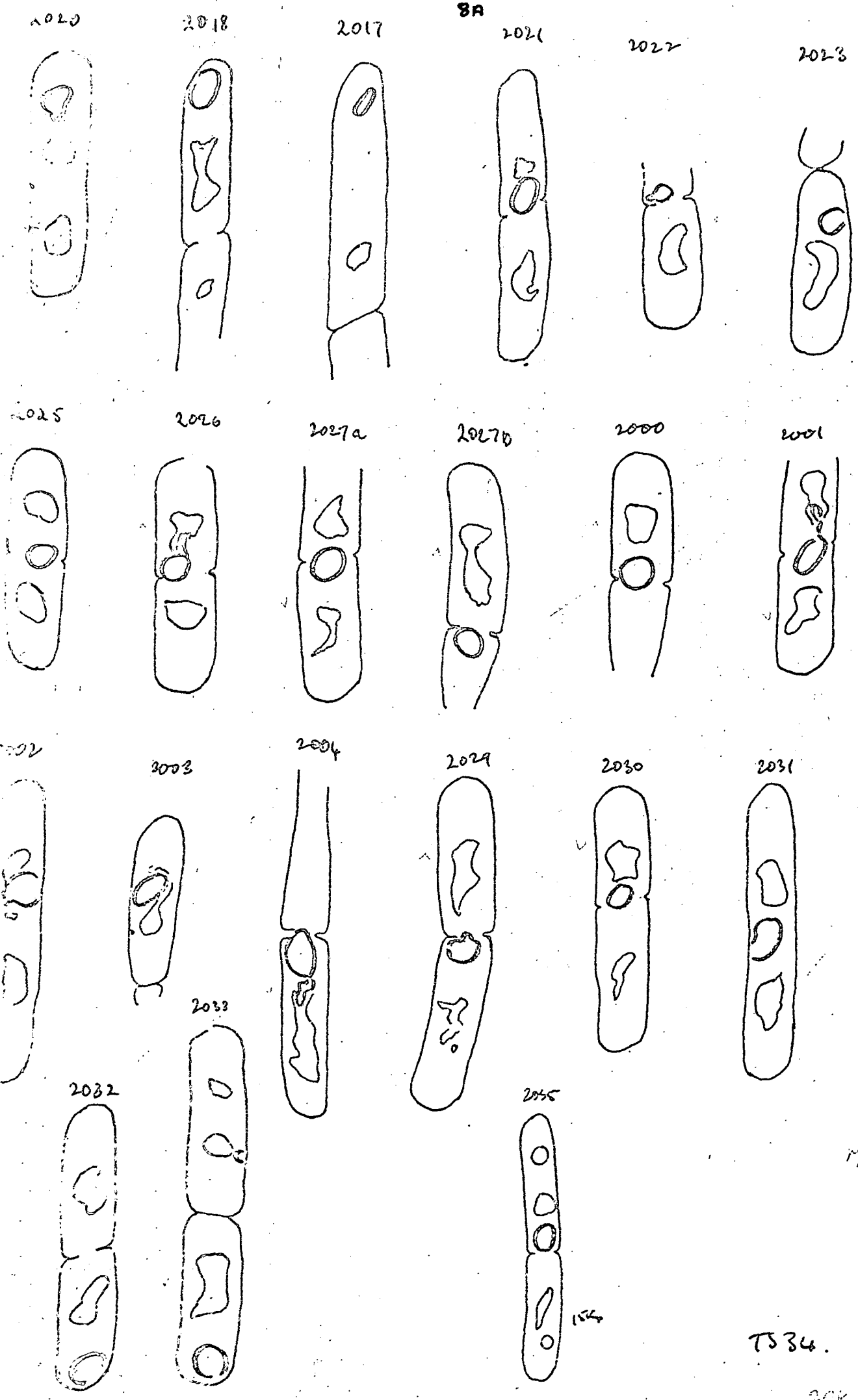
Transfer to outgrowth medium after 8 hours did not result in significant vegetative outgrowth, and, while

a few free cells were seen after 20 min., they were all abnormal (Plate 15), and generally became disintegrated later (Plate 16). The overall appearances at 30 and 60 min. were of spores arrested in the final stages of germination (Plate 17), although membranes appeared to become more lamellar (Plate 18); Figure 5 shows the distribution of the various kinds of membrane collections encountered, and indicates a change from vesicles to lamellae with time. True mesosomes were not observed, however, and any change observed may be artifactual.

Figure 5. Membrane development in alanine-inosine buffer. Spores germinated 8 hours and transferred to outgrowth medium. Selection criteria: good overall preservation; a region of cytoplasmic membrane in vicinity of membranes; approximately median section; an obvious membrane structure, of adequate size.

Time in Outgrowth Medium	Membranous structures observed		
	Lamellar	Vesicular	Mixed Ves. + Lam.
30 min.	9 (8%)	35 (33%)	63 (59%)
60 min.	54 (33%)	12 (7%)	100 (60%)

Germination in L-broth: Germination in a rich medium allows the cell to immediately commence macromolecular synthesis, and in the absence of biochemical data on its inception in the experimental system studied, there must be uncertainty in identifying physical states with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2023
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2001
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2035
154

MW.
2/5.

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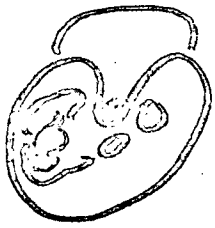

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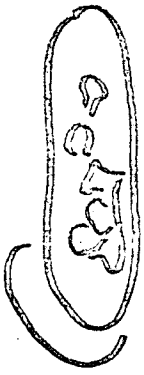

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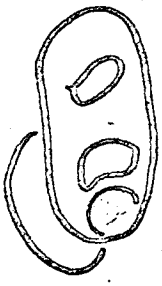

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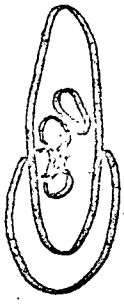

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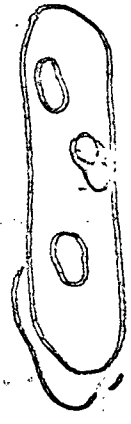

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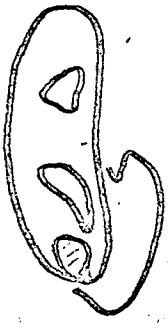

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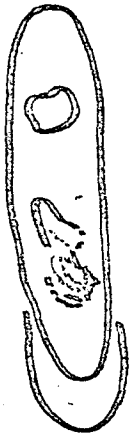

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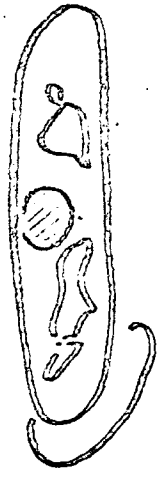

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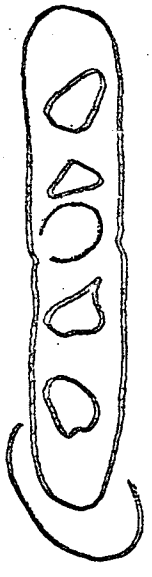

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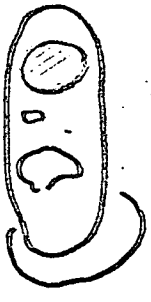

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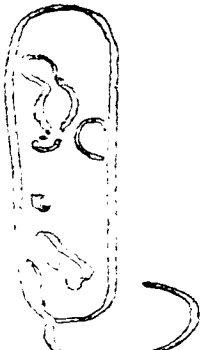

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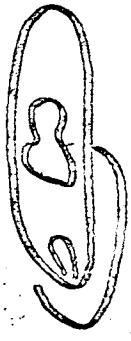


w. d. Indus. Alc
.35, 2m. N-limit.

similar ones observed in the nutrient deficient germination medium. Generally, however, germination appeared to take the same course (Plates 19-22) except that true lamellar mesosomes were observed in a significant number of cells before vegetative outgrowth commenced (Plate 21); their frequency was not analysed on account of the proviso above. Vegetative outgrowth occurred in a manner similar to that in alanine-inosine buffer, the cell growing through an area of dissolved spore integuments. Such cells were, however, much more like a normal vegetative cell, and at two hours some completely free cells were present, although most vegetatively outgrown cells had a spore remnant attached to one pole (Plate 24). At $2\frac{1}{2}$ hours (Plate 25), the number of free vegetative cells was greatly increased, some of which were dividing, and at 7 hours (Plate 26) the culture consisted almost entirely of free cells. Empty spore cases were infrequent at $2\frac{1}{2}$ hours, and hardly ever seen in sections from 7 hours, suggesting that they rapidly dissolved during vegetative outgrowth. Mesosomes were prominent in the three populations studied, taken at 2 hours, $2\frac{1}{2}$ hours, and 7 hours, but only a small proportion were entirely lamellar, most consisting of a mixture of vesicles and lamellae.

Mesosome distribution during outgrowth and early divisions in L-broth: Distributions were obtained from measurements of tracings drawn directly from negatives

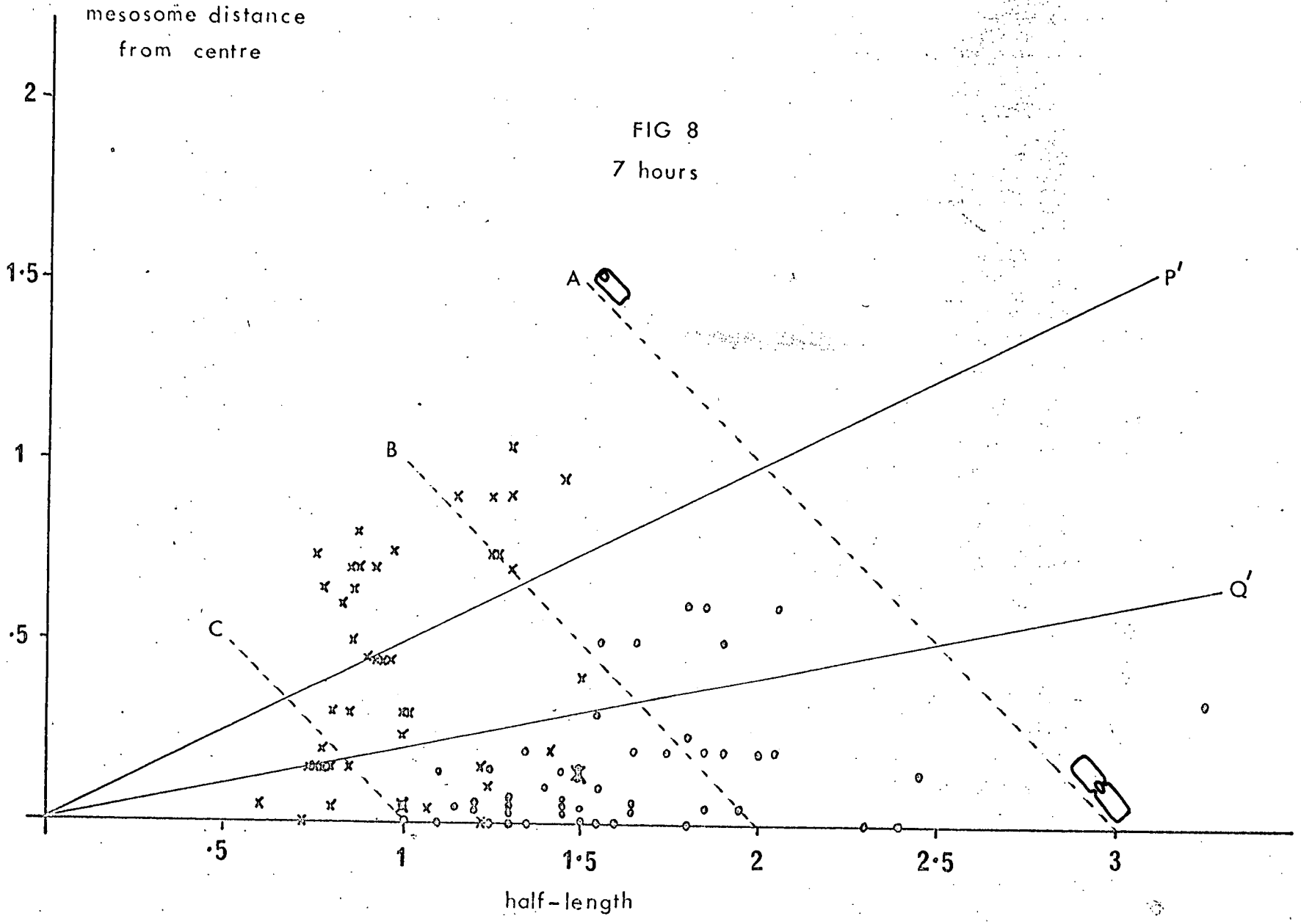
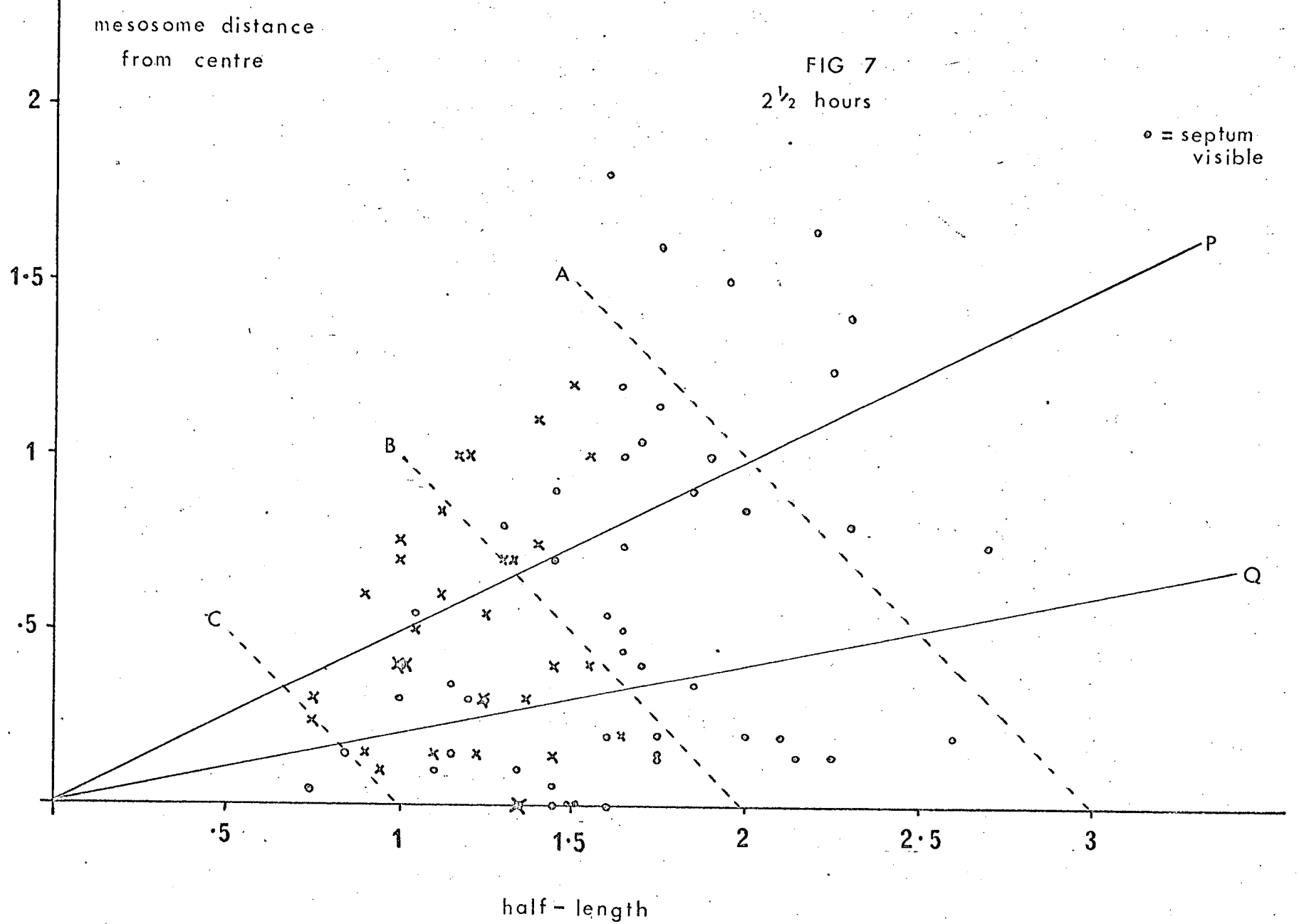

mesosome distance
from centre
2
1·5
1
·5
A
B
C
P′
Q′
·5
1
1·5
2
2·5
3
half-length

FIG 8

7 hours


mesosome distance from centre
FIG 7
2½ hours
o = septum visible
2
1·5
1
·5
P
Q
A
B
C
·5
1
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2·5
3
half - length

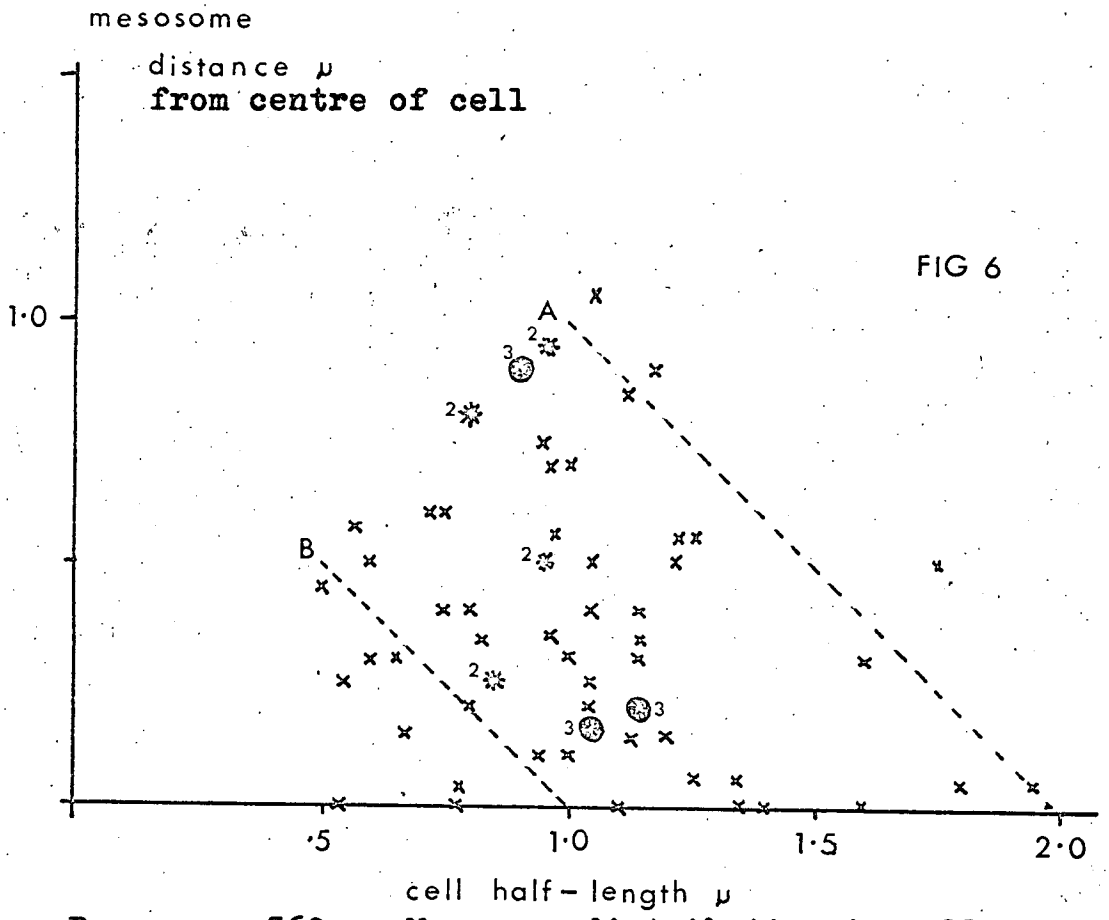


FIG 6

B. cereus 569. Mesosome distribution in cells outgrowing in L-broth after 2 hours incubation of spores. Sections selected for the presence of a spore remnant, and considered first-generation cells. Septating cells were excluded.

such as shown in Figures 6a, 7a, and 8a. Figure 6 shows the distribution, according to the method of Highton (1969) of mesosomes in vegetative cells with spore remnants still attached at 2 hours incubation and therefore considered to be the first generation. The cell half-length has been plotted along the x-axis, and the distance of the mesosome from the centre of the cell along the y-axis. Points are scattered similarly to the distribution reported in exponential cells; although the range in cell length is less, there is still more than a factor of 2 difference between the shortest and longest cell, this however being expected on account of the cell's origin from a small spore. The lines A, B show the behaviour of mesosomes in cells of initial lengths 2 and 1 u respectively if all the conditions of Highton's model were fulfilled.

Figures 7 and 8 show similar plots of mesosome distributions in cells without spore remnants, taken after 2½ hours incubation and therefore considered to be second generation cells, and after 7 hours for early exponential growth. The predicted behaviour in ideal cells of initial lengths 3, 2 and 1 u are given by the lines A, B and C on Figure 7 and 8. Points are given for septating and non-septating cells; consideration of the shortest cells with and without septa suggests that septum-building occupies about half of the cell cycle. The lines P and P′ therefore are the transition points of all cells from a septum-free to a septum-initiated

state at mid-cycle, assuming that septum-building occupies a constant fraction of the cell cycle in all cells, and that the cell grows exponentially. Ideally, all cells below this line will have visible septa, all cells above being free of septa. The lines Q and Q' are the transition lines for septum building during the last fifth (approximately) of the cell cycle.

The distributions in Figures 7 and 8 both show scatter; the range of cell lengths is increased, and a shift has occurred in Figure 8 such that mesosomes are now more often associated with septa as compared with Figure 7.

Discussion

Little is known about the origin of mesosomes in relation to biochemical events occurring during germination and outgrowth. There is fairly clear evidence that mesosomes are involved in sporulation (Fitz-James, 1969) and perhaps enclosed directly with the spore protoplast (Highton, to be published), but the physical state of membranes in spore protoplasts is not known, and the permeability of spores to fixatives may be very low (Rode, 1962). A study by Rousseau (1966) of *B. subtilis* spores did not show clear evidence of mesosomes during germination, and in any event it may be difficult to decide whether their appearance is related to macromolecular synthesis or not. Whilst the germinated spore may have defective protein-synthesising

ability (Idriss, 1969; Bleyman, 1969), the time scale for its resumption may be very short: 4 min. after germination in B. cereus T (Rodenberg, 1968). Additionally, whilst specific germinants in low concentrations may not support vegetative growth or outgrowth, certain outgrowth functions may proceed using endogenous materials (Setlow, 1970).

The results of the germination in alanine-inosine buffer suggest that certain membranous structures are indeed contained within the spore, but their relationship to fully developed mesosomes is not established. Further, any improvement in the membranous content or structure would appear likely to be due to endogenous metabolism, perhaps at the expense of ribosomes, as reflected in the poor ribosome content of cells that managed to establish vegetative outgrowth. This view is also substantiated by the very low viability of germinated cells transferred to outgrowth medium after a long time. It is certain, however, that in a rich medium in which outgrowth can ensue true mesosomes are established before vegetative outgrowth commences.

The distribution of mesosomes in outgrowing cells has been found to be very similar to that previously reported in established exponential phase cells (Highton, 1969), and therefore the heterogeneity of such cultures is established on germination (Fig. 7). It is not possible to conclusively decide whether the mesosome is randomly sited in the spore, if it does exist in an

intact form, nor is there any data for this experimental system on whether the spore encloses a cell with a completed replication cycle; thus, inclusion of partially replicated DNA (Ephrati-Elizur, 1971) may significantly alter cell topography during outgrowth and re-create the heterogeneity existing before sporulation. Heterogeneity could also arise if the mesosome were randomly placed in the spore, but subsequently on outgrowth the cell divided when the mesosome was centrally placed irrespective of the cell length at the time. The increase in the association frequency of mesosomes with septa at 7 hours (Fig. 8) suggests that eventually the culture behaves according to the model presented, where the mesosome divides and the cell septates as constant conjunctions of events, but that this occurs in a population of highly heterogenous initial cell lengths. Further, it is clear that the estimate of septum-building time calculated from short cells is inaccurate, and Figure 8 demonstrates that, assuming a constant fraction of the cell cycle, a good fit is obtained with the one-fifth transition line. Thus it is concluded that anomalous behaviour of the mesosome in relation to septation occurs in the first generation after outgrowth but subsequently the distributions fit well in very early exponential growth with the model of mesosome behaviour described by Highton (1969) as applied to a population containing a continuum of initial cell lengths.

SECTION III

Mesosomes of *B. cereus* 569

SECTION III

Introduction

The existence of intracytoplasmic membrane inclusions (mesosomes) has been confirmed in a large number of Gram-positive organisms (review, Ryter, 1969). There is no clear evidence of a common function for these organelles; however, there is circumstantial evidence for their involvement in a number of processes: for example, respiration (Reaveley, 1969), nuclear division (Ryter, 1964) and cell septation (Imaeda, 1963). In part, the lack of firm agreement on any function or set of functions is due to the considerable differences in their structure and number in the cell reported from different laboratories. Thus, Highton (1969, 1970) showed that B. licheniformis 749c and B. subtilis 172 contained a single lamellar mesosome when fixed with osmium-cyanide; different results for B. subtilis 168 ind were obtained by Ryter (1964) using osmium alone where there were several mesosomes per cell, and their structure vesicular. The influence of fixation technique on mesosome structure is well recognised (Ryter, 1969; Burdett, 1970), and Highton (1969) demonstrated that breakdown into vesicles, eversion, and redistribution of mesosomal membranes occurred in B. licheniformis 749c if the cells were left to stand before fixation. Such observations suggest that variations in mesosome content and structure may

represent fixation artifacts, the most likely *in vivo* condition for Gram-positive cells (rods) being the presence of a single lamellar mesosome.

B. cereus 569 cells treated in a way identical to that which demonstrated single lamellar mesosomes in *B. licheniformis* and *B. subtilis* have now been shown to possess several lamellar mesosomes, the lamellar structure being a variable possibly related to the age of the culture, but unrelated to the growth medium or growth rate.

Materials and Methods

Organism: *B. cereus* 569 (penicillinase-inducible) cultures were inoculated from a stock suspension of spores.

Media: Three media were used:

i) L-broth (casamino-acids 10 g/L, NaCl 10 g/L and yeast extract 5 g/L).

ii) L-broth supplemented with 0.05 M phosphate (as KH_2PO_4) and 0.002 M magnesium (as $MgCl_2$).

iii) The medium of Pollock (1963) containing trisodium citrate 5.9 g/L, ammonium sulphate 2 g/L, casein hydrolysate 10 g/L, glucose 2 g/L, gelatine to 0.1%, with 0.05 M phosphate and 0.002 M magnesium.

25 or 50 ml cultures were used, inoculated with 0.05 or 0.1 ml volume of a primary culture grown to a low optical density (approx. 0.1). All cultures were shaken at 75 cpm in a 37° water-bath, in stoppered

conical flasks of flask to culture volume ratio 5:1. Growth curves were constructed from optical density measurements at 620 mu using 1 cm. path-length glass cells.

Electron Microscopy: Twenty ml samples were removed at appropriate O.D.s and immediately pre-fixed with 2 ml osmium-cyanide fixative (Highton, 1969) at room temperature. After thorough mixing, the prefixed cells were centrifuged down, and resuspended in 1 ml volumes of osmium-cyanide fixative, after which 0.1 ml of 1% tryptone in 0.5% NaCl was added and the cells left to fix overnight at room temperature. Next day, 10 ml R-K buffer (Ryter, 1968) was added to each, the cells spun down and embedded in agar. Thereafter, the cells were stained with uranyl acetate, dehydrated in graded acetone steps, embedded in araldite and sectioned as described previously. All sections were post-stained with lead citrate, and viewed on an Elmiskop 1A at negative magnifications 20,000 x. Sections used for analysis were chosen for clarity of plasma-membrane round the whole of the cell and good overall preservation. For randomly chosen cells, at least six consecutive sections were allowed between samples to exclude serial-section bias, and measurements were taken from tracings drawn directly from negatives. Mesosomes were considered septum-associated if any part of the mesosome lay within 0.5 u of a line joining the septal edges.

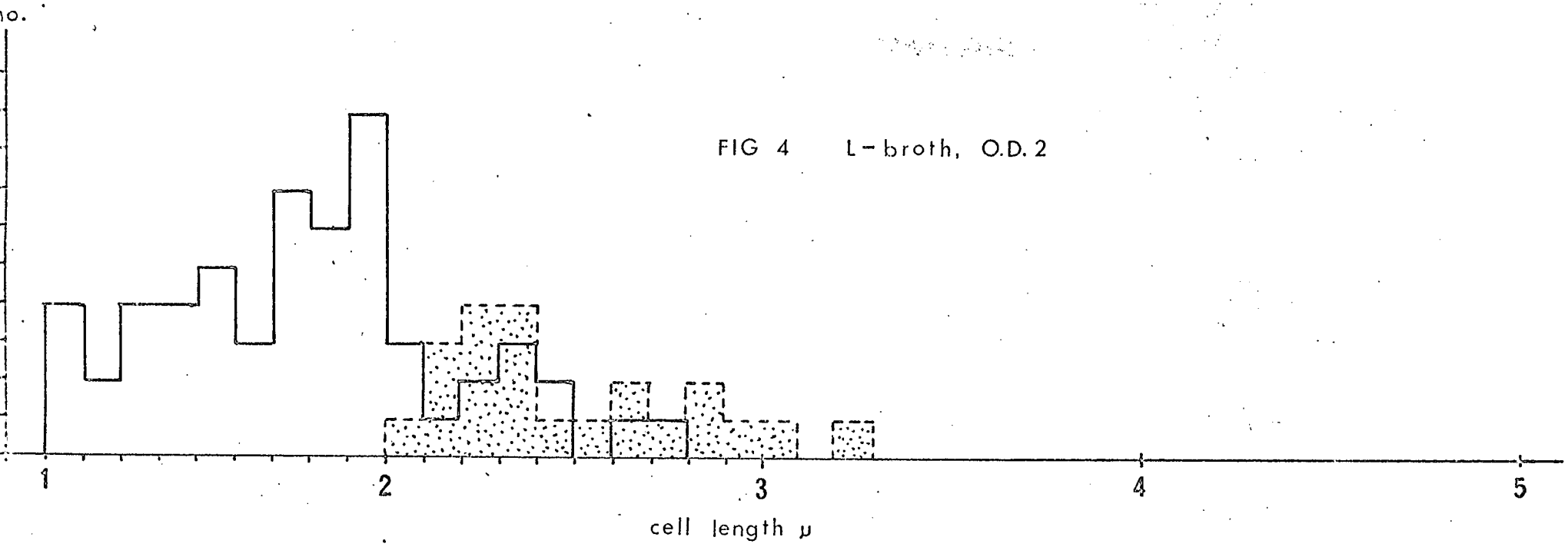


FIG 4 L-broth, O.D. 2

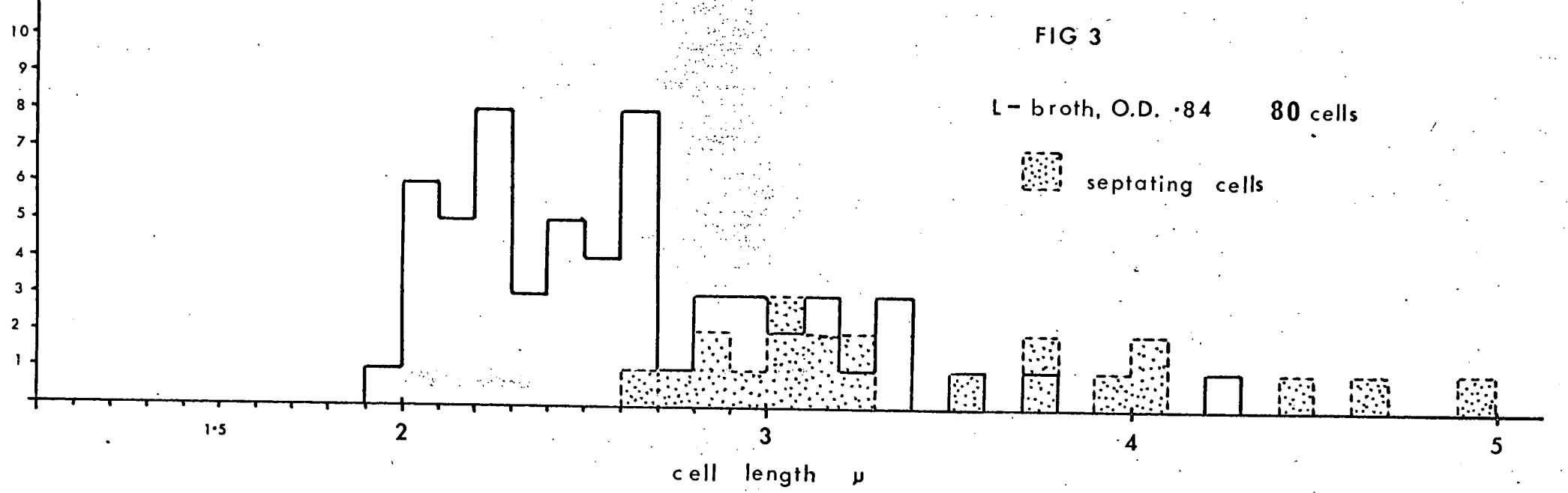


FIG 3

L – broth, O.D. ·84 **80** cells

septating cells

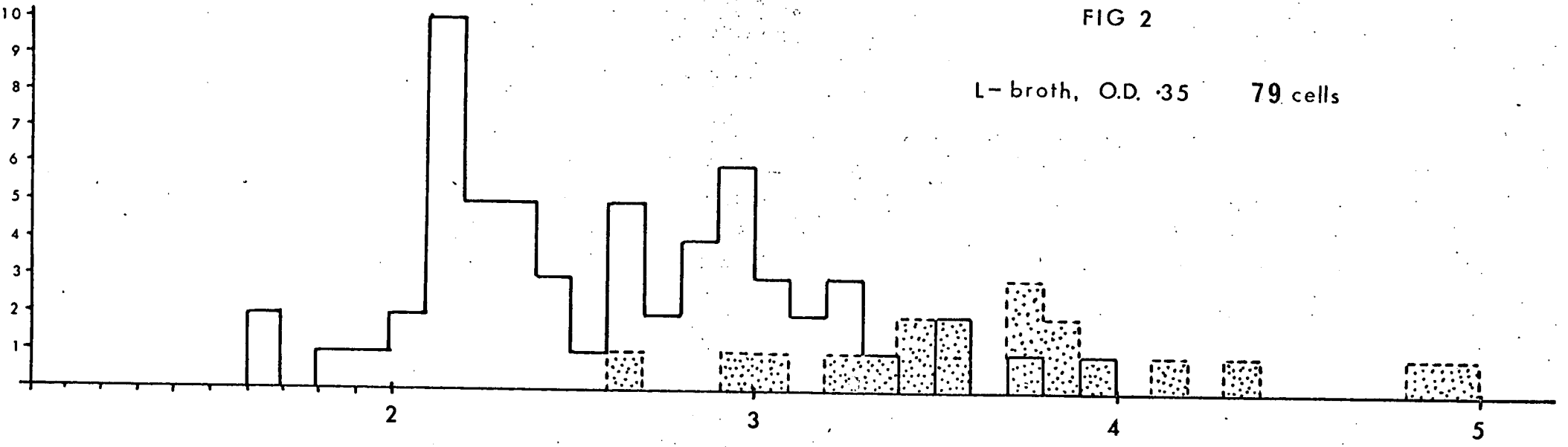


FIG 2

L – broth, O.D. ·35 **79** cells

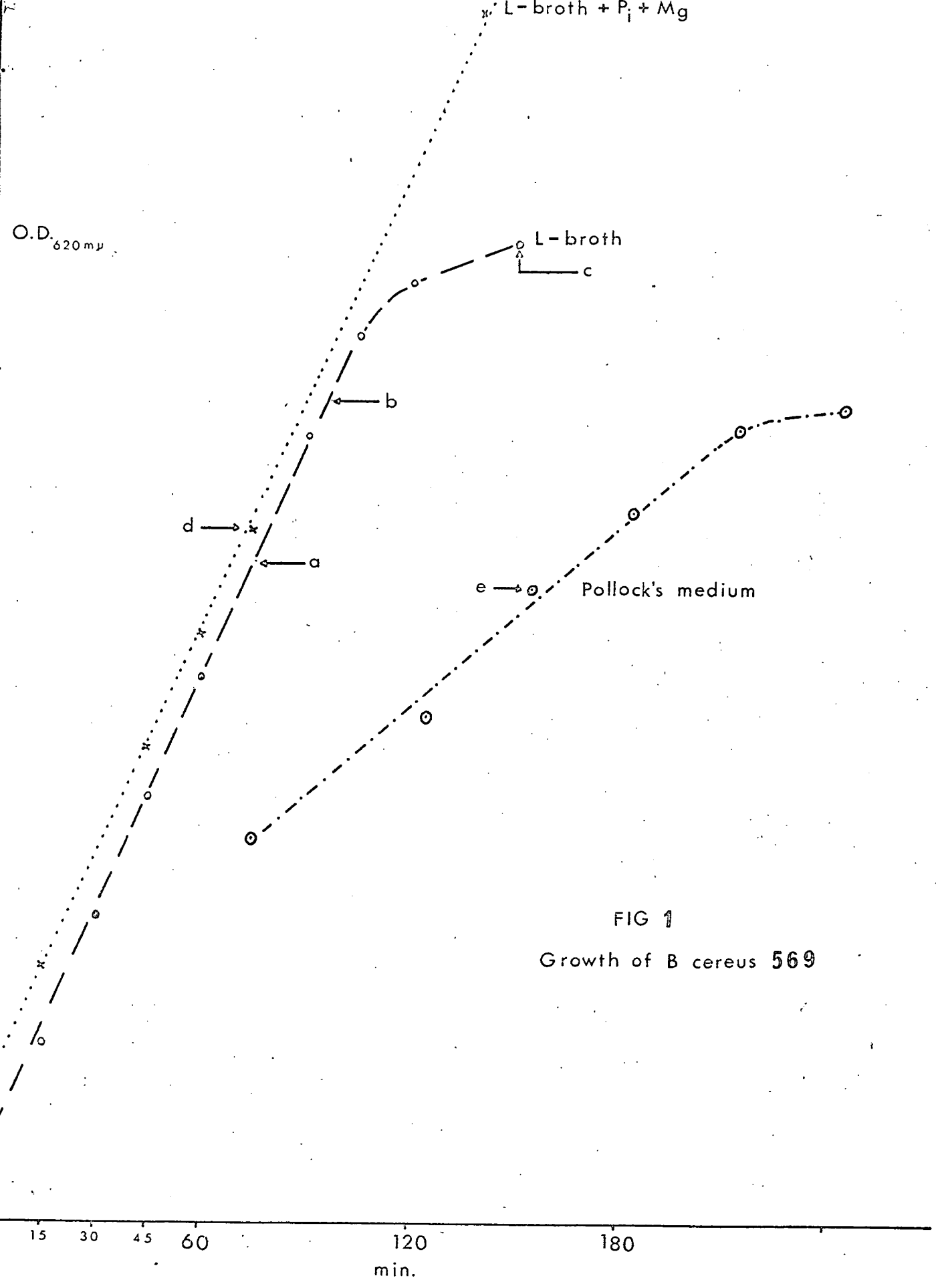


FIG 1

Growth of B cereus 569

Results

The mass doubling-times were 17, 17 and 32 minutes respectively for L-broth, supplemented L-broth and Pollock's medium (Fig. 1). Exponential growth continued longest in supplemented L-broth, and was shortest in Pollock's medium.

Mesosomes in L-broth

Samples for sectioning were taken at O.D.s 0.35, 0.84 and 2.0, at points corresponding to a, b and c on the growth curve. Each sample was taken from a different experiment. Plates 27-38 are representative of the sections studied, Plates 31-38 being a ~~group of three~~ cells at O.D. 2.0 serially sectioned. Figures 2, 3 and 4 show length distributions of dividing and non-dividing cells.

General cell morphology

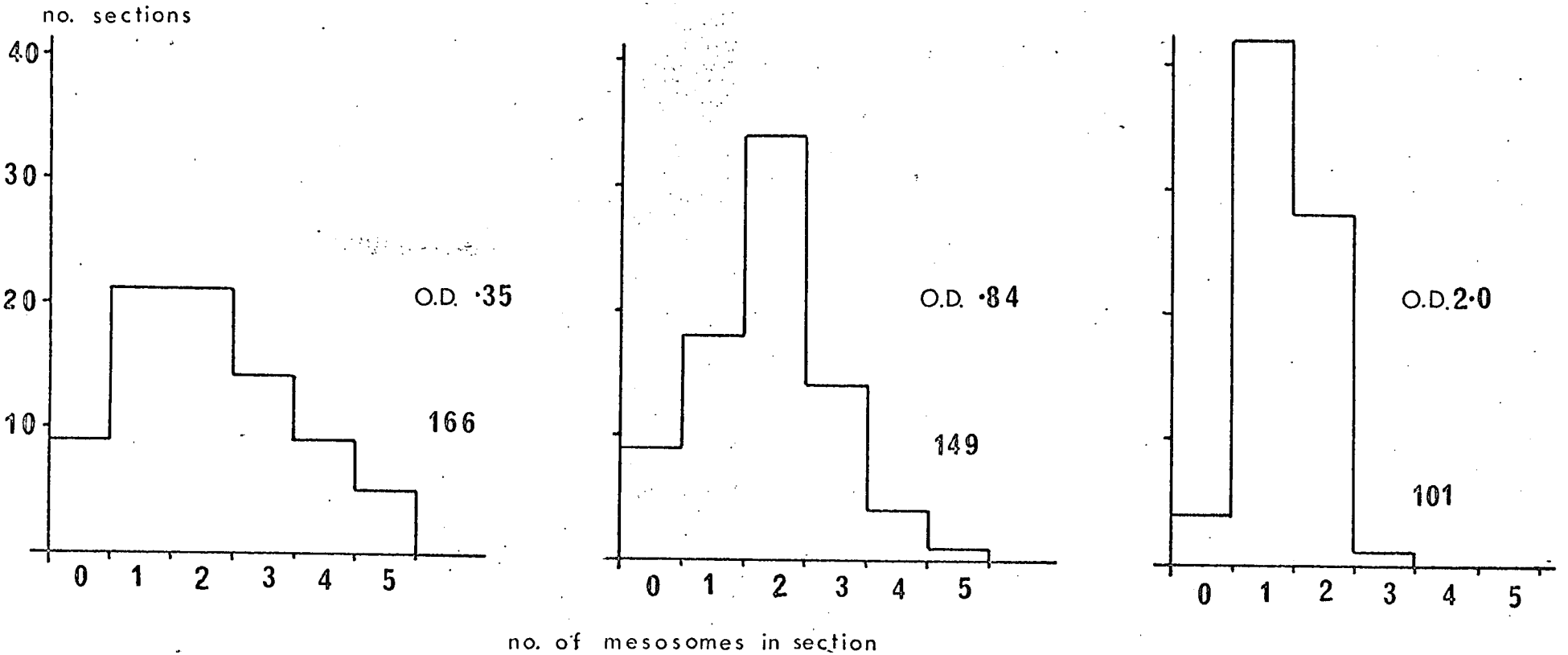
The general electron-microscope morphology of B. cereus 569 in section is similar to other Gram-positive rods. Features distinguishing this organism from B. licheniformis 749c in section are a wall without obvious lamellation, a plasma membrane, typically tri-laminate, but wavy in outline, and DNA areas generally scattered throughout the cytoplasm (Plates 27 and 28). Most significantly, more than one mesosome is present in both dividing and non-dividing cells; mesosomes are contained within membranous sacs probably derived from

the plasma membrane, and their structure is variously vesicular, lamellar or a mixture of both. A high proportion appear also to have physical contact with DNA areas.

a) Mesosomes in cells at O.D. 0.35: Mesosomes in cells at this O.D. were of mixed type and generally circular in section with diameters of .1 - .3 u. Lamellar mesosomes consisted of concentric triple-membranes approximately 80 Å from edge to edge separated from each other by variable gaps of between 80 and 160 Å. Entirely vesicular mesosomes were infrequent, most non-lamellar mesosomes containing mixtures of vesicles and lamellae. Vesicles varied greatly in size and a unit-membrane was often difficult to discern, most appearing to be bounded by a single dark line and filled with amorphous material. Additionally, in a few cells with entirely vesicular mesosomes, the outer layer of the plasma-membrane was highly stained and very irregular (Plate 29). This increased contrast of the plasma-membrane was not seen in any cells with lamellar mesosomes, even if vesicular or lamellar-vesicular mesosomes were also present elsewhere in the cell.

Data were collected from 79 randomly chosen axial sections; 19 of these cells had incomplete septa. The shortest non-dividing cell was 1.6 u long, the longest 3.9; corresponding lengths for dividing cells were 2.6 and 4.9 u (Fig. 2). Mean lengths were 3.6 and 2.6 u

FIG 5 L-broth cells



Mesosome distribution in cells grown in L-broth and sampled at the O.D.s shown. Figures on the R. of each histogram show the total number of mesosomes in the sample.

for the respective classes. A total of 166 mesosomes were counted, of which 36 were mixtures of vesicles and lamellae or vesicles only, the remainder being entirely lamellar. 48 mesosomes of all types were physically associated with nuclear material, and of the 19 septating cells, 9 had a mesosome associated with a septum. Septating cells (24% of the population) contained 41% of all mesosomes, but only a sixth of these mesosomes were associated with the septum (Fig. 10). The mesosome distribution is given in Figure 5. The average mesosome content per section was 2.1.

b) Mesosomes in cells at O.D. 0.84 (Plate 30): Mesosomes were almost all entirely lamellar, with no structural differences from those at the lower O.D. Neither the mean cell lengths nor the proportion of dividing cells in the population was significantly altered (Fig. 3). The proportion of all mesosomes in dividing cells was reduced to 27%, but more of these mesosomes were associated with septa, viz. 30% (Fig. 10). The average mesosome content per section was 1.9 (Fig. 5). No cells with dark plasma-membranes were observed.

c) Mesosomes in cells at O.D. 2.0: Such cells were beyond exponential phase (Fig. 1). Nearly all mesosomes were entirely vesicular or lamellar-vesicular and were generally slightly larger, most being 0.2 - 2.5 u dia. All cells had a dark plasma-membrane. Data were obtained from 79 randomly chosen axial sections and from 11 serially-sectioned cells (Plates 31-39). For the

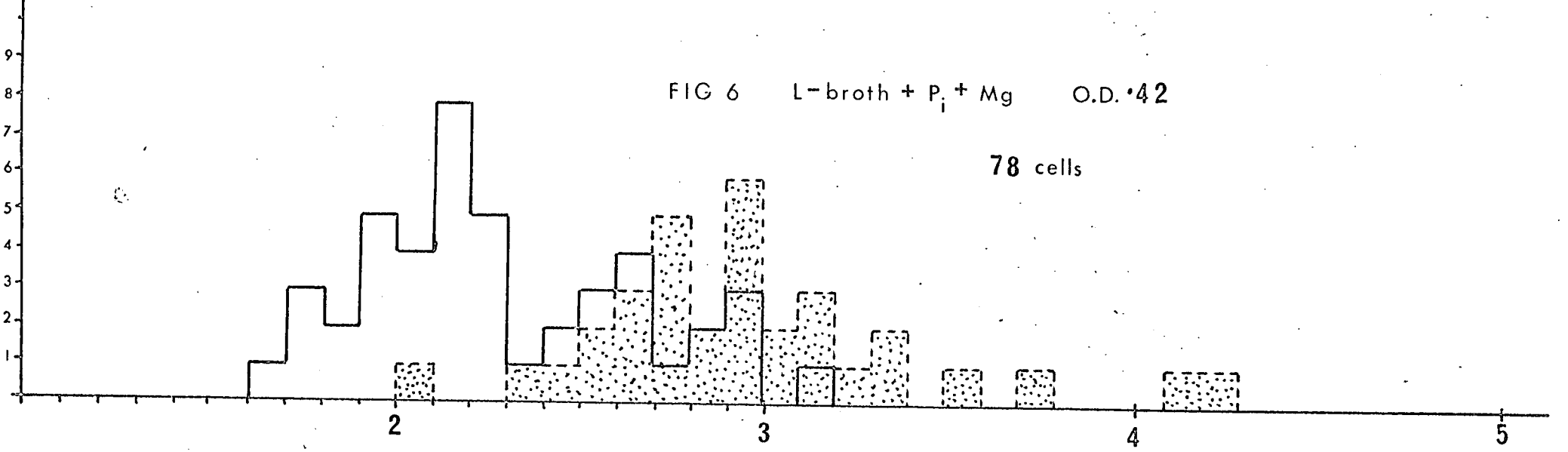


FIG 6 L-broth + P_i + Mg O.D. ·42

78 cells

cell length μ

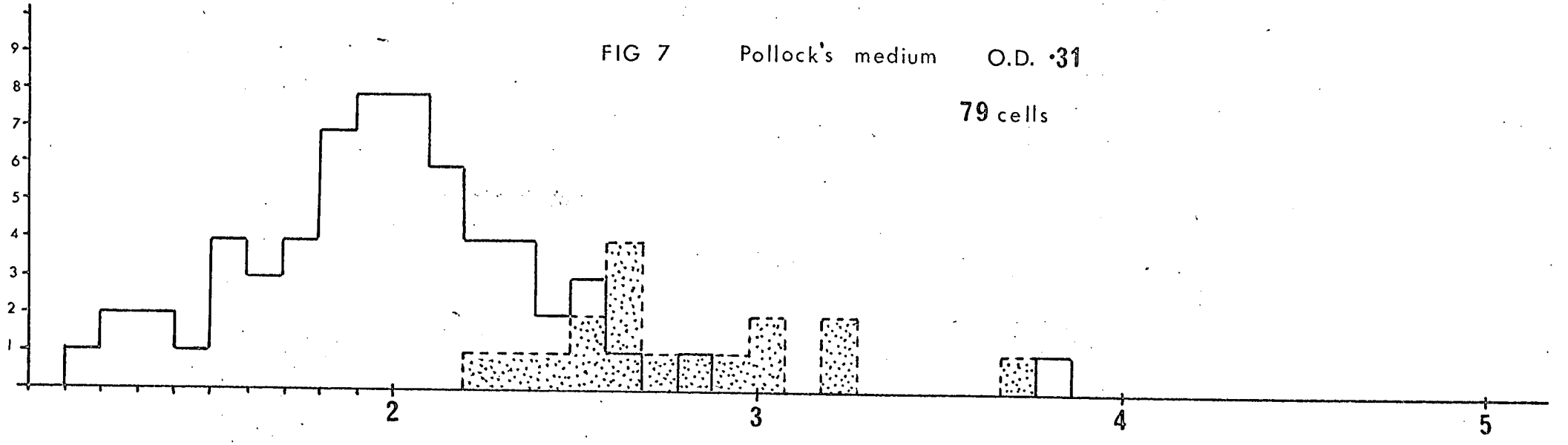


FIG 7 Pollock's medium O.D. ·31

79 cells

randomly chosen population, the mean cell lengths were reduced (1.7 and 2.4 u) but the proportion of septating cells remained unchanged at 28% (Figs. 4 and 10). 32% of all mesosomes were contained in septating cells, 70% of which were associated with septa. The average mesosome content per section was 1.3 (Fig. 5). Of the 10 serially-sectioned cells considered complete, all had more than 1 mesosome; generally, four of the central sections could all be considered axial on the criterion of visibility of the plasma-membrane round the cell, and a correction factor of 1.8 could be applied to axial sections to convert the average number of mesosomes per section to the average number of mesosomes per cell. Two septating cells were serially sectioned; one did not have a mesosome associated with the septum, the other had two opposite each other at the septum edges but not in contact.

Mesosomes in supplemented L-broth and Pollock's medium

Specimens were taken at O.D.s 0.42 and 0.31 respectively at points d and e on the growth curve (Fig. 1). Structurally, the mesosomes in both were almost entirely lamellar (Plates ~~40~~ 39, ~~41~~ 40) and without significant differences. Mean cell lengths were slightly shorter than those in L-broth at an O.D. of 0.35 (Figs. 6 and 7). The average mesosome content in supplemented L-broth was higher than in Pollock's medium, and the proportion of mesosomes in dividing cells and

Figure 10

Mesosome distribution in B. cereus 569 cells

O.D. and Medium	Mean cell lengths u		% Dividing cells	Average mesosome content/ section	% all mesosomes in dividing cells	% mesosomes associated c. septa in dividing cells	% septations c. mesosome(s)	% lamellar mesosomes
	Non-dividing	Dividing						
L-broth 0.35	2.6	3.6	24 (19/79)	2.1	42 (70/166)	15 (11/70)	46 (9/19)	79
L-broth 0.84	2.6	3.4	26 (21/80)	1.9	27 (40/149)	30 (12/40)	47 (10/21)	97
L-broth 2.0	1.7	2.4	28 (22/79)	1.3	32 (33/101)	70 (23/83)	90 (20/22)	3
L-broth & Pi; + Mg 0.42	2.2	2.8	40 (33/78)	1.9	47 (70/148)	27 (19/70)	45 (15/33)	95
Pollock's Medium 0.31	1.8	2.8	21 (17/79)	1.4	26 (28/110)	18 (5/28)	30 (5/17)	95

Figures in parentheses denote absolute numbers

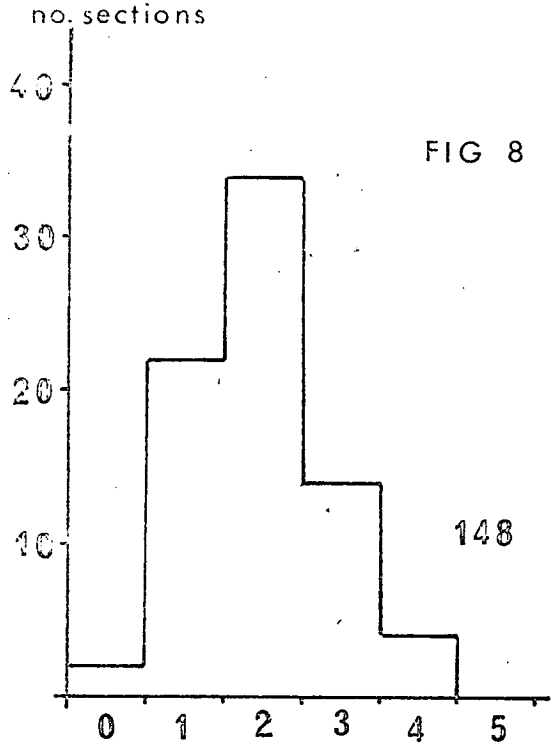


FIG 8 L-broth + P_i + Mg

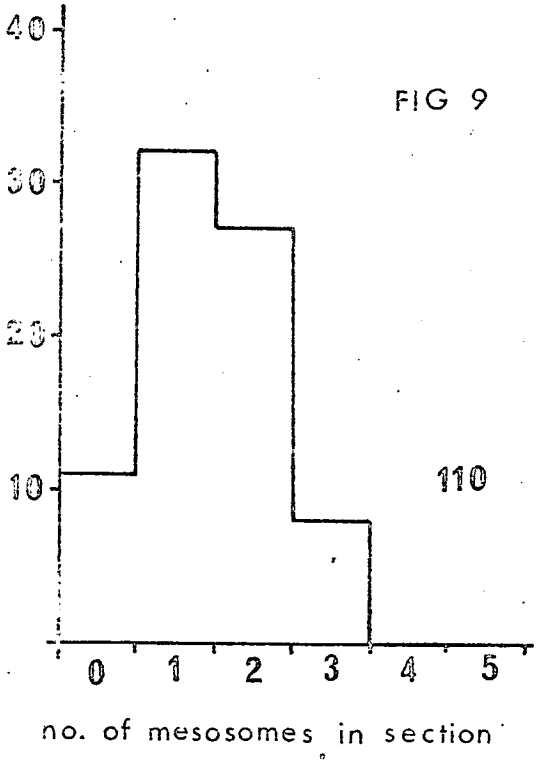


FIG 9 Pollock's medium

Mesosome distribution in cells grown in supplemented L-broth and Pollock's Medium.

Figures on the R. of each histogram show the total number of mesosomes in the sample.

associated with septa, and the proportion of septations with mesosomes, also higher (Figs. 8 and 9).

The analysis of all the populations is summarised in Figure 10.

Discussion

Certain conclusions can immediately be drawn from the results presented. Firstly, B. cereus 569 cells contain several mesosomes, and, secondly, their structure, whether lamellar or vesicular, is not a function of growth medium. A similar conclusion on the effects of growth medium and rate on the mesosomes of B. subtilis was reached by Ryter (1969) although this organism is there reported to have several vesicular mesosomes per cell in direct contrast to the evidence for a single lamellar mesosome given by Highton (1970). Assuming that the number and structure of mesosomes in cells of different strains of the same species are similar, such large discrepancies are difficult to reconcile. It is clear, however, that in B. cereus 569 cells, lamellar and vesicular forms of mesosomes occur and occasionally co-exist. The results in L-broth suggest that culture age is significant in relation to mesosome structure; inspection of Figures 5 and 10 show that the transition from exponential to stationary phase is accompanied by not only a change from lamellae to vesicles, but, additionally, mesosomes have a much higher association frequency with septa, their spread in

distribution being reduced. Also, stationary phase cells had dark plasma-membranes and all cells with dark plasma-membranes had entirely vesicular mesosomes; however, there were some exponential phase cells with normal membranes which had vesicular mesosomes. Changes in mesosome structure and number as cells move into a sporulation state are a recognised phenomenon (Ryter, 1964; Gould, 1970), and presumably these changes observed in B. cereus 569 are pre-sporulation events, and coupled with a significant reduction in the average mesosome content.

Various attempts have been made to ascribe function to mesosomes. Mesosomal association with septa has been widely recognised; for example, Ellar (1967) proposed a septum-building function in B. megaterium from observations made on a synchronised culture. It is not possible to say conclusively from these results that some septa in exponential growth do not have an associated mesosome, but the probability of this being so is small; thus, considering a single mesosome of 0.1 u diameter at a septum of a cell of 0.7 u diameter, and assuming that 8 out of 10 sections needed to serially section the cell will pass through cytoplasm and not just wall, then the probability of this mesosome appearing in any section is about 0.25. In fact, the proportion of septations with a mesosome at the septum approaches 0.5, suggesting that septal mesosomes are larger or extend round the septum, or that there are a number of them at the septum. It

is clear, however, that only a small proportion of mesosomes in early exponential dividing cells are septum associated, the mesosome positions gradually being reorganised as growth progresses to produce the very high association with septa described above. In fact, the method of cell synchrony employed by Ellar produced presporulation cells, and, given a similar mesosome structure and behaviour in B. megaterium as in B. cereus 569, Ellar's conclusions on such an isolated population would be quite understandable.

Another suggestion is that mesosomes are involved in nuclear division (Ryter, and Jacob 1964). Whilst this is less attractive in cells that contain a single mesosome which divides after the DNA has segregated into the daughter halves, association with the DNA of a high proportion of mesosomes in B. cereus 569 (about 30% irrespective of the population except in stationary phase in L-broth) makes this proposal a distinct possibility.

No evidence for a role in teichoic acid synthesis was obtained from the results in supplemented L-broth; phosphate and magnesium lack, as exists in normal L-broth, has been shown to significantly alter teichoic- and teichuronic-acid ratios in the wall (Ellwood, 1970), but no change was detected in the mesosomes if these ions were supplied.

The existence of many mesosomes in a cell makes it impossible to construct a model for their behaviour as the cell grows and divides. Despite their association

with septa, no electron-microscope evidence was obtained suggesting that they divided, as in B. licheniformis and B. subtilis, and it should be added that nearly 50% of all septations encountered could be considered asymmetrical on the basis of one daughter half exceeding the other by greater than 0.1 u. This of course may be artifactual but represents a difference compared with sections of B. licheniformis. The alternative for their behaviour would thus appear to be that new mesosomes are synthesised as the cell grows and divides, and it may be concluded that with the demonstration that these cells contain several lamellar mesosomes in three different media, while other species contain only one, the possibility now demonstrably exists of species-specific mesosomes with functions unique for each species.

SECTION IV

Mesosome development in *B. cereus* 569 germination and the effect of metabolic inhibitors

SECTION IV

Introduction

The previous study of mesosomal origin in *B. licheniformis* 749c germination suggested that mesosomes, or precursors in the form of vesicles, were conserved in the resting spore, but that development of their exponential-phase morphology might require certain outgrowth functions. Difficulty of interpretation arose through the poor germination response of the spores to specific germinants in a nutrient-deficient medium in which the macromolecular synthesis initiated in normal outgrowth would probably be largely inhibited. Using a rich medium, it was established that mesosomes could be fully developed in pre-emergent cells and that the cell lengths and mesosome distributions in the first few division cycles were similar to those in an exponential-phase population, demonstrating that a continuum of cell lengths was produced on vegetative outgrowth. Whilst the first division cycle showed anomalies, the subsequent mesosome behaviour accorded well with the model presented by Highton (1969) of a single lamellar mesosome dividing with the cell.

B. cereus 569 exponential cells have more than one mesosome (Sect. III), and their spores were found to germinate rapidly with specific germinants. These observations firstly suggested that the mesosome system may be different from *B. licheniformis*, and, secondly,

allowed investigation of its origin in germination and of any requirement for macromolecular synthesis in its development by using chloramphenicol to inhibit protein synthesis and Actinomycin D to inhibit RNA synthesis during subsequent outgrowth in a rich medium. A possible outcome of such investigations, not realised, was the use of a germination system for specific autoradiography of developing mesosomes with a view to chemically characterising mesosomal membranes so identified and extruded from spore protoplasts.

The results show that some ill-defined membrane structures are visible in germinated spores but that full mesosomal development requires outgrowth and an external nutrient supply; further, new RNA synthesis is required in the first 30 minutes of outgrowth to develop at least a proportion of mesosomes, but once this has occurred further inhibition of RNA synthesis leads to the apparent production of extra membranous material possibly of mesosomal origin.

Materials and Methods

Spores: B. cereus 569 spores were prepared from a 600 ml 36-hour culture in S-broth (peptone 10 g, Lab-Lemco (Oxoid) 2.4 g, NaCl 2 g, water 1 l.) aerated at 37°C. Spores were harvested by centrifugation, washed with approximately equal volumes of sterile distilled water three times, and then concentrated to about $\frac{1}{25}$th of the original volume. After a further 3 washes, the

suspension was layered onto a 60% sucrose solution, 80 ml per 20 ml spore suspension, and spun at ~~2500 rpm~~ 1,600 g for 15 min. Pellets contained a high spore density on phase contrast microscopy, and after a series of 6 washes, the suspension was heat-treated at 60° for 1 hour. The pellet remaining after 4 final washes was resuspended and kept at 4°C.

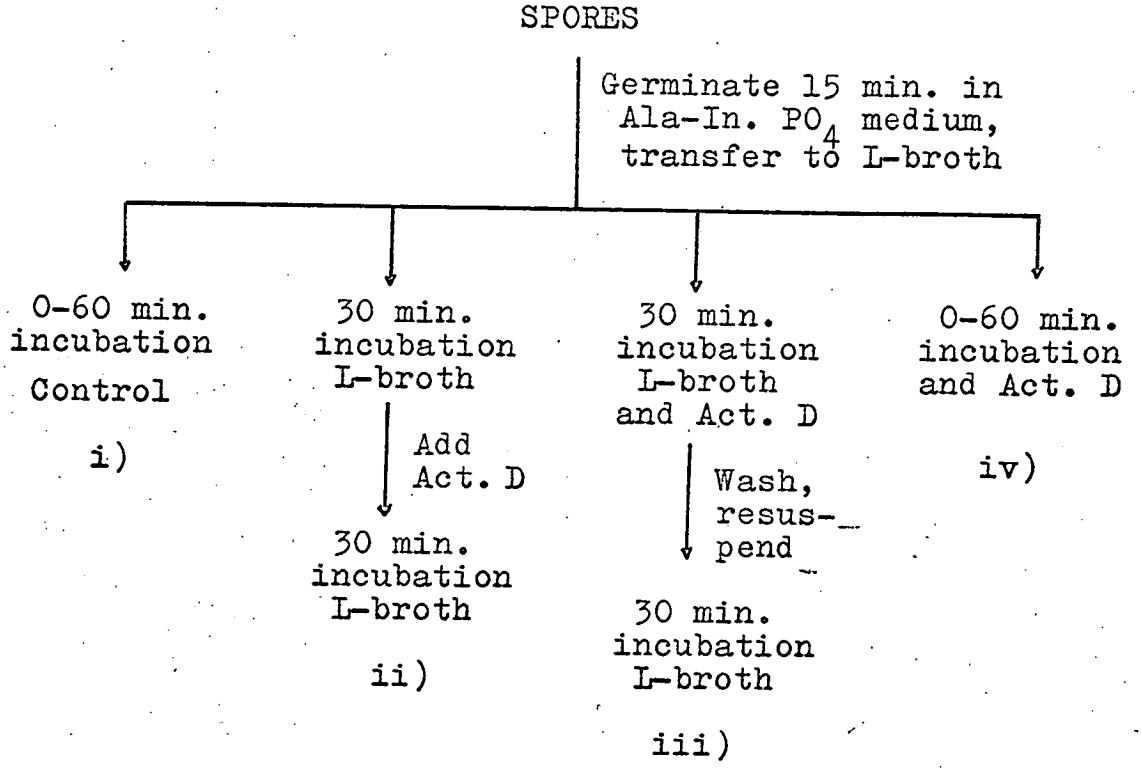
Germination: Spores were germinated in a medium containing 0.01 M L-alanine and 0.01 M inosine in a 0.01 M sodium phosphate buffer pH 7. One or two ml of the spore suspension was centrifuged and resuspended in 10 ml germination medium; after 15 min. static incubation at 37°, the spores were centrifuged again for resuspension in a rich medium in outgrowth experiments, or directly pre-fixed for electron microscopy before centrifugation.

Outgrowth: Four experiments were performed. The first established normal mesosome development during outgrowth in L-broth, and the succeeding two investigated the effect of chloramphenicol (CAP) and Actinomycin D (A.D.) on outgrowth in L-broth and residual membrane development in nutrient-deficient medium after a period of "priming" in L-broth. Finally, the distribution of tritium from labelled acetate was examined in fractions of outgrowing cells.

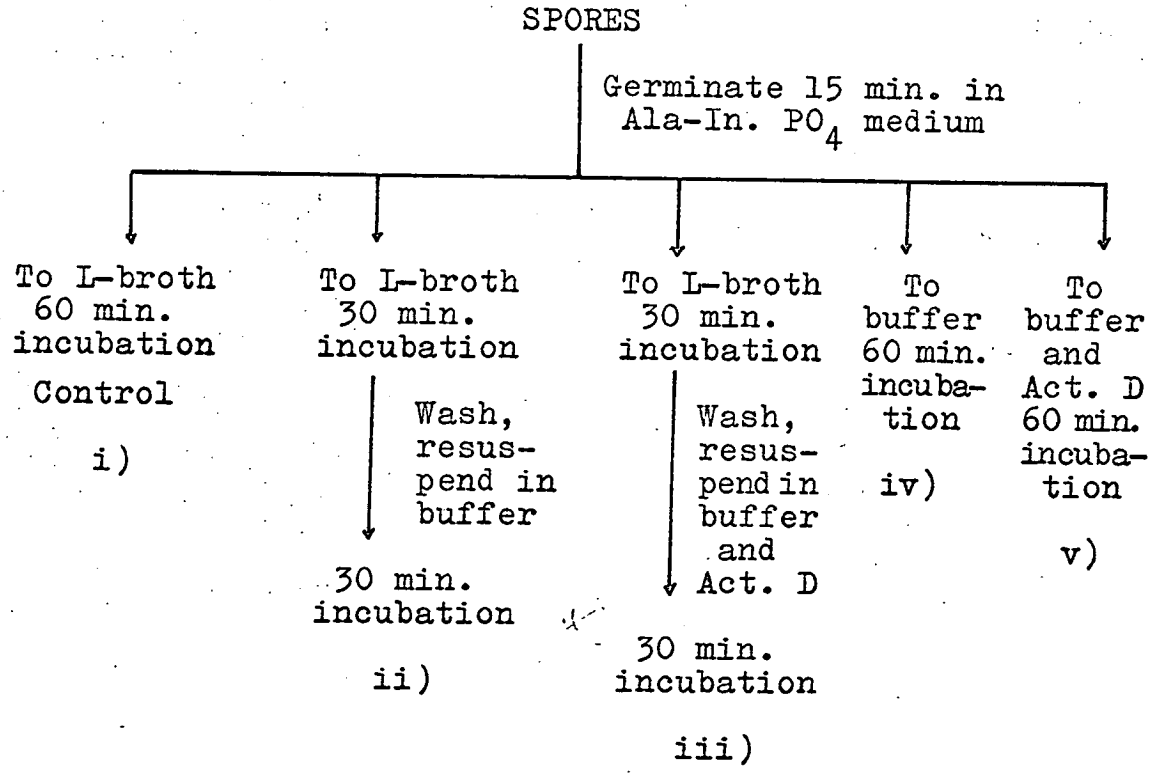
a) Outgrowth in L-broth: After germination and centrifugation, spores were resuspended in L-broth and incubated for 1 hour before being pre-fixed and fixed for

FIGURE 1

a) Rich medium experiment (L-broth)



b) Buffer experiment



electron microscopy.

b) Effect of CAP and A.D. on outgrowth in L-broth:

i) CAP: After germination, equal spore quantities were pipetted into 4 flasks containing L-broth, to 3 of which CAP had been added to a final concentration of 200 ug/ml. The control and one of the CAP-treated cultures were fixed after 1 hour's incubation, and the second CAP-treated culture fixed at 2 hours. The last CAP-treated culture was grown for 30 min., washed twice with fresh medium and resuspended in fresh medium without CAP for a further 30 min. incubation before fixation.

ii) Actinomycin D: A similar scheme was followed; one culture received Actinomycin D (A.D.) in the first 30 min. of outgrowth only, a second in the second 30 min. of outgrowth only, and a third throughout the whole hour of outgrowth. All cells were therefore fixed at a total incubation time of one hour. Figure 1 shows a flow-diagram of the procedure.

c) Effect of A.D. on cells primed for outgrowth but exposed to nutrient lack: After 30 min. outgrowth in L-broth, cells were spun, washed and resuspended in 0.01 M Tris-HCl buffer pH 7 containing 1 mg/ml sodium acetate and incubated 30 min., one culture containing A.D. As controls, cells were incubated for 1 hour in acetate-supplemented buffer with and without A.D., without being primed by 30 min. incubation in L-broth. This scheme is also shown in Figure 1.

All incubations were carried out at 37°C in flasks

of volume 5 x that of the culture, shaken at 75 cpm. The concentration of A.D. was in all experiments 2 ug/ml.

d) Distribution of ^{3}H-labelled acetate in cell fractions: After germination, two 1 ml aliquots of concentrated spores were incubated for 30 min. in 10 ml L-broth containing 2 uc/ml C-1-labelled tritiated acetate. One culture was then pasteurised at 60° for 10 min. to kill the cells, cooled and washed 3 times with 0.01 M Tris-HCl buffer pH 7 containing 1 mg/ml medium acetate carrier. The other culture was rapidly cooled, centrifuged and washed 3 times with fresh "cold" medium and incubated a further 30 min. without isotope followed by pasteurisation and washing in buffer. 0.38 g EDTA was added to both, and the suspensions exposed to a total of 20 min. ultrasound (6.5 u in 30 sec. bursts) to fracture the spore coats. Five ml of each sonicate was added to 5 ml 10% TCA and a precipitate allowed to form overnight at 4°C. This precipitate was centrifuged down at 4,000 rpm for 20 min., the supernates collected and the pellets extracted with 2 ml 75% alcohol at 45° for 30 min. Two ml water and 2 ml ether were then added, the mixtures thoroughly shaken and left to stand for separation. The lipid-containing ether layer was pipetted off, and both samples centrifuged again before collection of the aqueous alcohol layer. 0.1 ml samples of each layer (TCA supernate, ether and aqueous alcohol) were pipetted onto duplicate glass-fibre discs, and the total residue remaining washed onto filters with distilled water.

Filters were dried at 60°, and counted in 5 ml Dimethyl-POPOP in Dioxan on a Beckman scintillation counter.

Electron microscopy: All samples for electron microscopy were pre-fixed and fixed in osmium-cyanide, stained, embedded and sectioned as described previously (Sect. III).

All cell masses were determined by O.D. at 620 mu in 1 cm path length glass cells, and viabilities were assessed from plating out 10-fold dilutions in triplicate onto nutrient agar, incubated at 37°C; a pasteurisation step of 60° for 15 min. was applied in determining loss of heat resistance.

Results

The spore suspension contained over 99% phase-bright spores; almost all were surrounded by an exosporium resistant to lysosyme and EDTA in various concentrations. Spore bodies were approximately 0.5 u long by 0.3 u wide, mostly oval, and with some size variation. The concentration of the stock suspension was about 2×10^9/ml, and gave rise to colonies with a uniform morphology on plating of appropriate dilutions. In alanine-inosine-phosphate buffer, over 95% of spores had lost their phase-brightness in 15 min., and 96% of the population was ~~not~~ heat-sensitive; the reduction in heat-resistance was from 1.5×10^9 to 6×10^7 plating units/ml after germination. Individual spores followed under phase-microscopy germinated in between 40 and 90 seconds, the average being

about one minute from complete phase-brightness to complete phase-darkness.

a) Mesosome development in normal germination and outgrowth: The dormant spore (Plate 41) has the essential characteristics of all bacterial spores; the spore protoplast is structureless in section, without a discernible plasma-membrane. The surrounding cortex, 500 Å wide, is also structureless, although there is a suggestion of a dark-staining inner layer, arrowed. The cortex is enclosed by a convoluted laminated coat about 300 Å thick in which a series of dark-staining lamellae are sandwiched between an inner amorphous layer of low contrast and an outer amorphous layer of high contrast. A wide space separates the spore proper from the exosporium (Ex). The spore core is roughly oval, and corresponds to the area of phase-brightness.

The prime events in germination are the appearance of intracellular structure (ribosomes and DNA) and a plasma-membrane, and the cortex becomes distinctly layered and thinner (Plate 42). The spore core swells, and the convolutions of the coat disappear probably as a result of stretching due to the swelling of the protoplast (Plate 43). This swelling, however, does not apparently preclude a certain amount of wrinkling in the outline as shown in Plate 44. No change occurs in the dark-staining lamellae of the coats or the outer amorphous layer, and it is difficult to determine whether

the thinning of the cortex also involves the inner amorphous layer of the coat. True mesosomes were not seen in any germinated spores, but certain darkened areas were sometimes seen which could be membranous; mostly, such areas were indistinct (Plate 45) with a suggestion of lamination, and one case was encountered of indistinct vesicular structures lying underneath the plasma-membrane (Plate 46). Of 103 well-preserved sections cut centrally either transversely or longitudinally (distinction of axis cut was not always possible due to ovoid shape and size variation) 22 exhibited such dense areas with a striated appearance.

As outgrowth commences, the DNA becomes more compact and centrally placed, and mesosomes or membranous bodies become distinct (Plates 47 and 48). True mesosomes were usually lamellar-vesicular, and, in addition, the plasma-membranes were of the dark-staining kind described previously (Sect. III) in nearly all outgrowing cells. Using the same criteria as for germinated spores, and excluding all emergent cells, 52 out of 103 axial sections showed a true intracytoplasmic membranous structure after 60 minutes outgrowth. The process of emergence is indicated by the separation of the cortex into two layers and the localised dissolution of the spore coats (Plate 49); the inner layer of the separated cortex contains the dark layer visible previously, and is closely applied to the cell which grows out through the gap in the coats (Plate 50), and is thus presumably conserved wall material.

FIG 2

Effect of Chloramphenicol on exp. growth of B cereus 569 in L-Broth

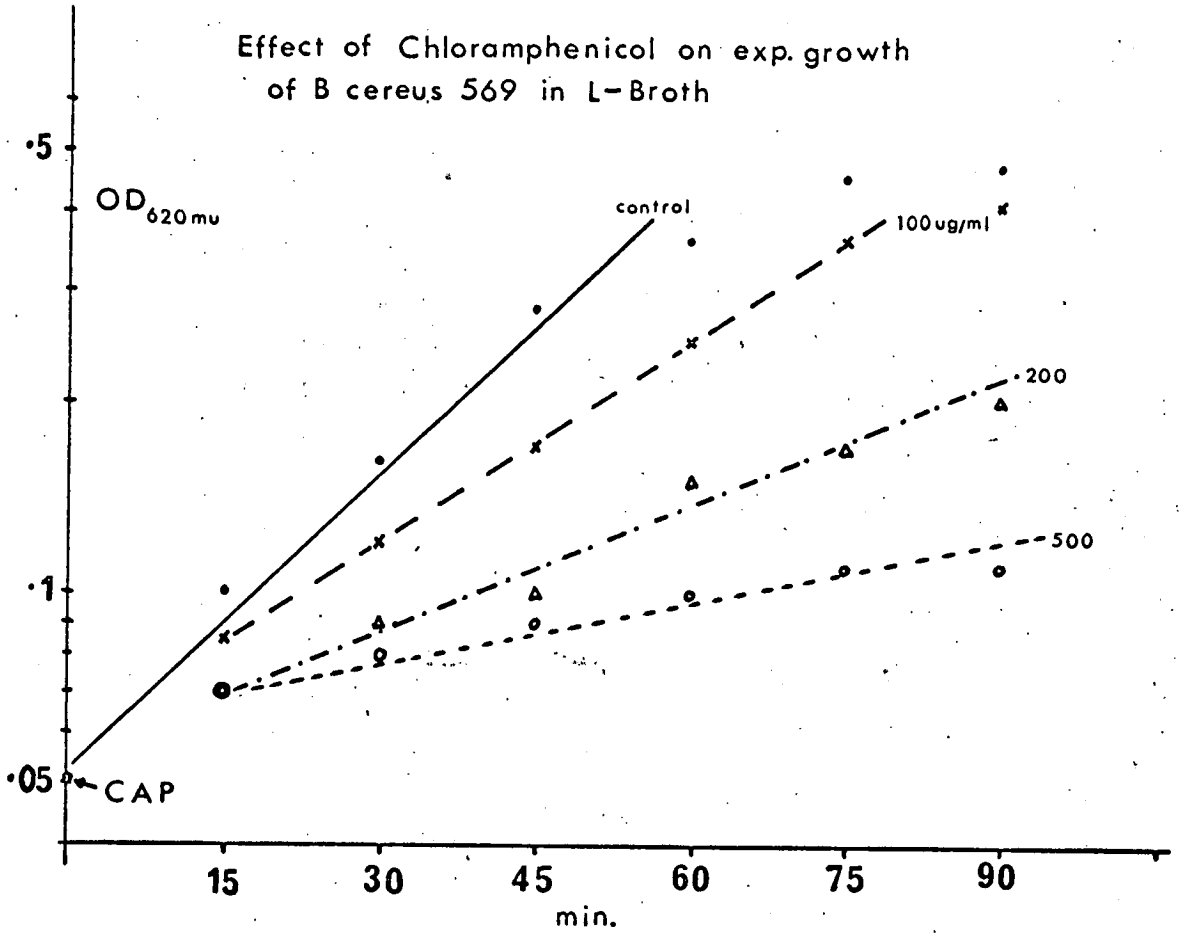
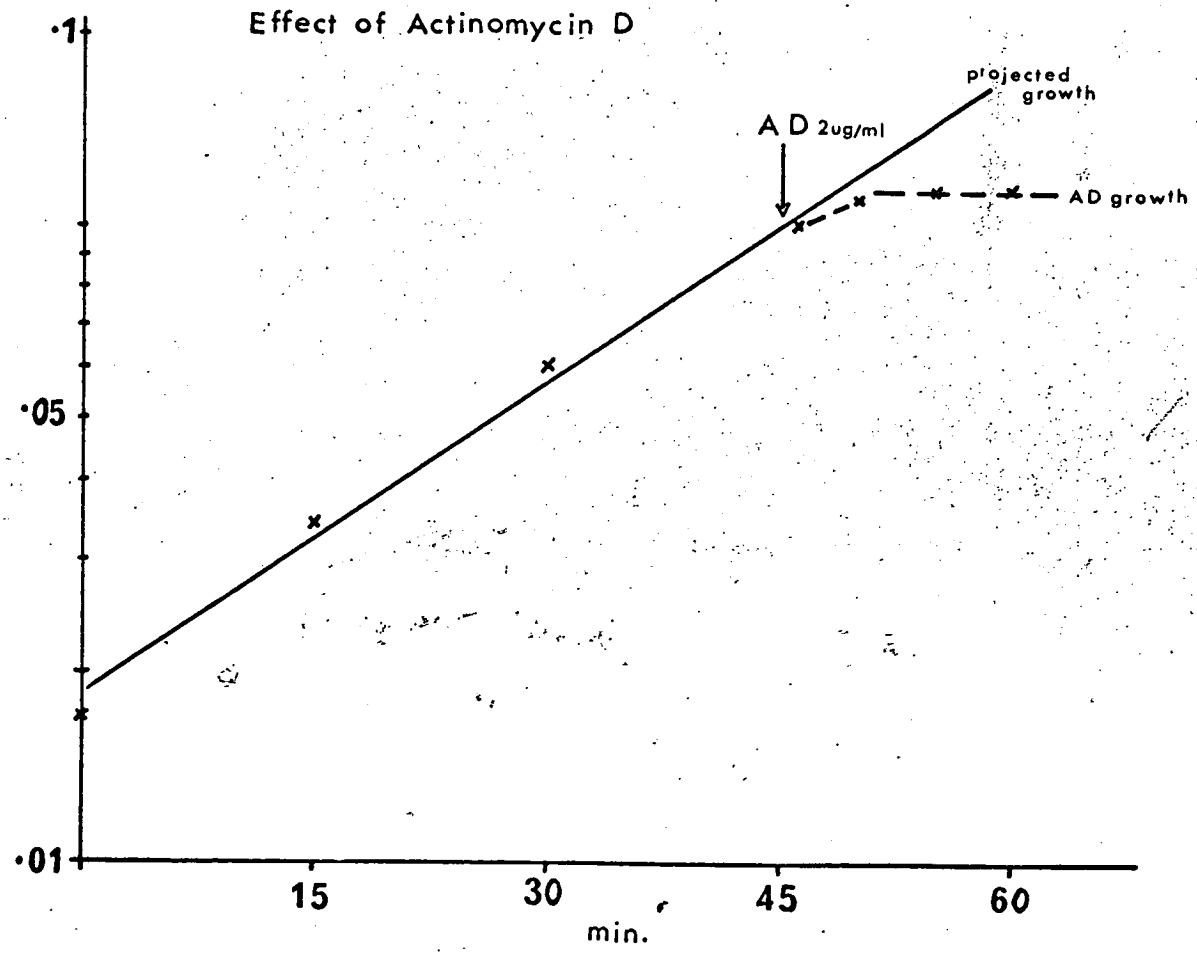


FIG 3

Effect of Actinomycin D



The eventual fate of the spore case appears to be complete solubilisation, as few remnants were visible by light microscopy in an outgrown culture. Finally, the number of true mesosomes in outgrowing and emergent cells was more than one in a significant number of sections (Plate 50).

b) Effect of CAP and A.D. on outgrowth in L-broth: The selection of the concentrations of these inhibitors used in the experiments was made on the basis of their effect on the exponential growth curves shown in Figures 2 and 3; these are discussed more fully in Section V.

i) CAP: The concentration of CAP used (200 ug/ml) produced an approximate doubling in the generation-time of exponential cells (Fig. 2). The only observable effects on outgrowth were a rounding-off of the DNA generally (Plate 51) and an apparent delay in establishing emergence after one hour's exposure to CAP. Mesosomes were often present and conspicuous in this sample.

ii) A.D.: Exponential growth was immediately halted by the concentration of A.D. used (2 ug/ml) (Fig. 3). Cells exposed to A.D. for one hour after germination appeared to have progressed very little from germination, although nearly 10% had membranous structures in axial sections (Plates 52, 53). Cells that were exposed firstly to A.D. for 30 minutes and then regrown for 30 minutes had progressed further, and about 25% of axial sections showed intracytoplasmic membranes that could be

Figure 4

Estimates of mesosomes during germination and outgrowth of *B. cereus* 569. Sections selected for good preservation and full visibility of plasma membrane.

Specimen[a]	Cells without membranes	Intracytoplasmic membranes	Extra membranes	Total
1 Germinated spores	101 (22 with dark areas)	1	0	102
2 60 min in L-broth	51	52	0	103
3 60 min in L-broth and AD	86 (24 with dark areas)	9	0	95
4 L-broth and AD followed by L-broth without AD	67*	25	1	93
5 L-broth, followed by L-broth with AD	38	30	33	101
6 L-broth, followed by buffer without AD	66*	34 (degenerate)	0	100
7 L-broth followed by buffer with AD	Mesosome breakdown, no extra membranes			
8 60 min in buffer	Regression to spore state			

* Includes cells with dark areas, breakdown not available.

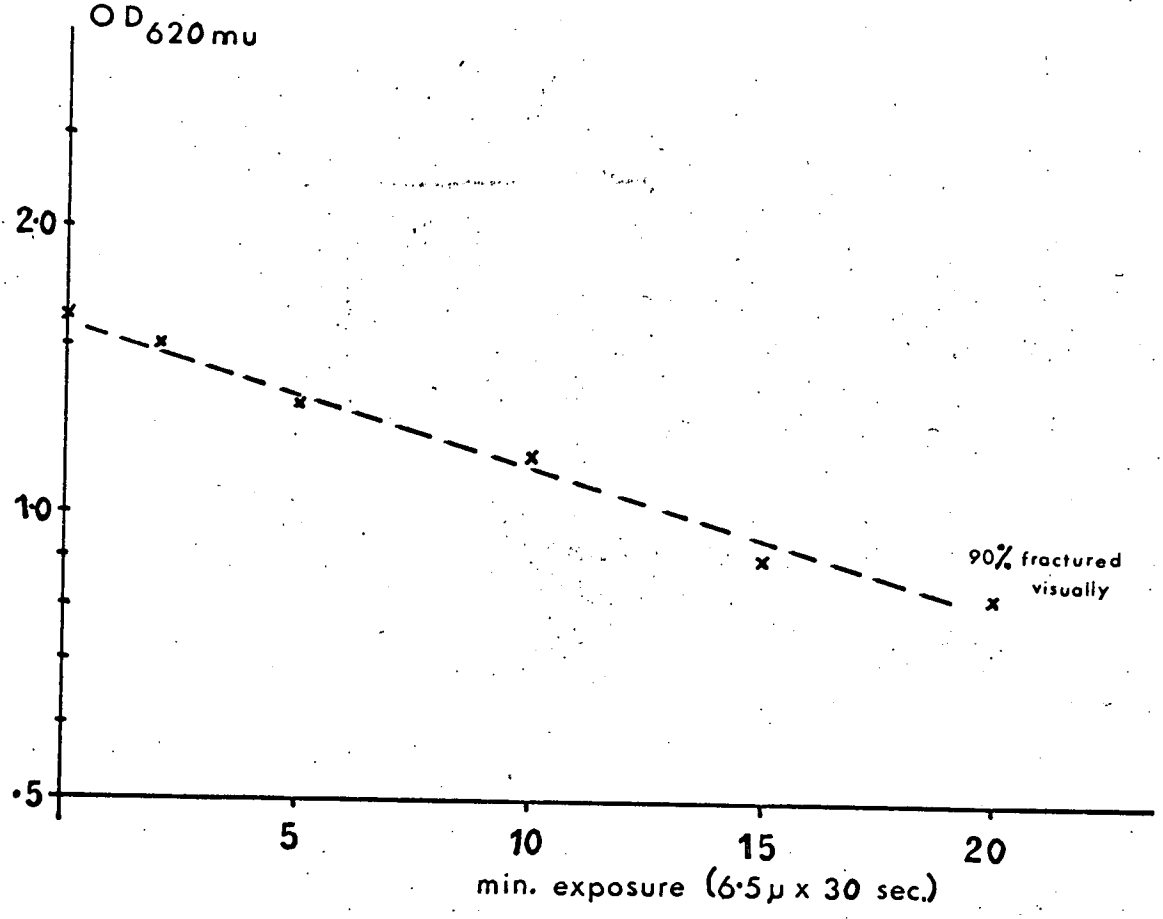
a) All spores were germinated for 15 min. in alanine-inosine-phosphate buffer.

considered mesosomes or their immediate precursors (Plates 54, 55). There were no emergent cells. Cells that received A.D. in the latter 30 minutes of outgrowth were generally at a similar stage of development, but many sections now showed extensive collections of apparently extra membranes lying between the plasma-membrane and the wall (Plate 56). In some cases (Plate 57) a mesosomal origin was indicated, and in others (Plate 58) a second attachment to the plasma-membrane was suggested. It was also clear that in some cells there were multiple layers of membrane completely encircling the plasma-membrane in section (Plate 59). The membranous material had very similar structure to lamellar mesosomal membrane, consisting of 2 dark layers separated by a light, the thickness being approximately 100 Å from edge to edge; additionally, the membranes were usually very wavy, and in this respect similar to dark plasma-membranes. Excluding cells with extra membranes whether a mesosome was present or not, a similar proportion of axial sections showed mesosomal structures as in the previous specimen; sections with extra membrane accounted for a similar proportion, and appeared to be correlated with a corresponding reduction in the proportion of cells containing darkened cytoplasmic areas (Fig. 4).

c) Mesosome development in buffer after priming: Mesosomes in cells grown for 30 minutes in L-broth and then resuspended in buffer with acetate were largely degenerate

FIG 5

O.D. regression on spore breakage with ultrasound



(Plate 60), and appeared in approximately a third of the sections. Fewer (about 10%) of cells treated with A.D. in the second incubation phase in buffer showed complete mesosomes (Plate 61), and these were usually highly degenerate with some small vesicles appearing between the plasma-membrane and wall (Plate 62). Cells resuspended in buffer directly after germination, with and without A.D., did not show any features of outgrowth, and none treated with A.D. appeared to be reverting to spores. In no specimen were extra membranes seen.

These results are summarised in Figure 4.

d) Incorporation of tritiated acetate: Sonication was estimated to be over 90% effective on the basis of reduction in O.D. and phase microscopy observations (Fig. 5). Figure 6 shows the distribution of tritium label in the various cell fractions examined. Counts are background subtracted.

Figure 6.

	Abs. counts/ filter	dpm/5 ml sonicate	Approx. % distribution
30 min. pulsed cells			
a) TCA supernate	28	2.8×10^3	43
b) Ether fraction	90	1.8×10^3	29
c) Aqueous alcohol fraction	19	7.6×10^2	12
d) Total residue	10^3	10^3	16
30 min. pulse followed by 30 min. chase			
a) TCA supernate	35	3.5×10^3	63
b) Ether fraction	55	1.1×10^3	20
v) Aqueous alcohol fraction	15	6.0×10^2	10
d) Total residue	400	4.0×10^2	7

Discussion

B. cereus 569 has a typical germination process on electron-microscope evidence. Many spores will germinate on contact with low concentrations of specific germinants especially alanine, inosine and phosphate (Foerster, 1966). Specific initiation factors for *B. cereus* strains have been described (Vary, 1968) and many lytic enzymes such as lysosyme will also induce germination (Fitz-James, 1971). It is uncertain whether cleavage of disulphide bonds in spore coats is a significant factor in germination (Setlow, 1969) but ultrastructural changes in the coats of *B. cereus* T on germination have been described (Hashimoto, 1971). *B. cereus* T apparently has a biphasic germination process related to the cations present (Hashimoto, 1969), and the earliest measurable event is the loss of heat resistance followed by calcium, dipicolenic acid and muco-peptide loss (Dring, 1971). Germination is considered complete when the spore is fully permeable and has lost its heat resistance; generally, this can be recognised microscopically by the ability to distinguish cytoplasmic contents and the thinning or disappearance of the cortex. *B. cereus* 569 shows these features, although the cortex is not lost completely on germination as in another *B. cereus* strain (Hamilton, 1967). The finding that true mesosomes, or recognisable intracytoplasmic membranes were absent in germinated spores would appear to agree with the pictures presented by Hamilton (1967)

and Moberley (1966) of other B. cereus germinated cells, but, nevertheless, the existence of certain dark areas suggests that some form of membrane structure is contained within the B. cereus 569 spore.

Outgrowth is defined as the commencement of macromolecular synthesis. In a number of organisms it has been shown that germinated spores are deficient in protein-synthesising ability (Sakikibara, 1969). B. cereus T germinated spores apparently have defective ribosomes (Kobayashi, 1965) and are devoid of m RNA, but this is remedied very early in outgrowth. Actinomycin D binds to DNA and prevents RNA polymerase from functioning, probably by a steric effect (Wells, 1970); the inhibition of normal mesosome development by A.D. in B. cereus 569 outgrowth suggests a requirement for new m RNA, the slight increase in the number of intracytoplasmic membranes over germinated spores supporting the view that precursors do exist in the spore and mesosomes may be able to develop in certain cases at the expense of endogenous materials (Sect. II). It is clear, however, from the split culture experiments in L-broth that once cells have commenced outgrowth, new membranes are synthesised in the presence of A.D., since more membranes were produced when A.D. was added after 30 min. growth in L-broth than were produced in 30 min. L-broth after being held in the germinated state for 30 min. by addition of A.D. This suggests that either the m RNA or the resulting enzymes are long-lived. Further, it is necessary for cells to

have an external nutrient supply for this A.D.-resistant synthesis to occur, as shown by the results in acetate buffer. Acetate would be available for some lipid synthesis, e.g. fatty acids, although if the TCA cycle is inoperative, as in B. cereus T (Blumenthal, 1965), phospholipid synthesis and energy metabolism would be drastically curtailed. The concept that mesosome development, and synthesis of new membrane, occurs partially at the expense of existing precursors is indicated by the decrease in cytoplasmic dark areas as the proportion of sections with obvious membranes rises (Fig. 4, lines 1 and 2).

Little conclusion can be drawn from the effect of CAP, as some residual synthesis was allowed at the concentration used; however, it is interesting to note that CAP inhibition in exponential cells follows 1st-order kinetics, i.e. is concentration-dependent. CAP does not usually behave as a competitive inhibitor, its effect being mainly to upset the ribosomal proteins and hence ribosomal function.

Finally, it seems clear that tritium derived from the C-1 of ~~glycerol~~ acetate is widely distributed, and thus unlikely to provide a specific membrane label. A similar technique was used successfully, however, to label membrane synthesis during sporulation of B. cereus T (Fitz-James, 1969).

Thus, it has been demonstrated that outgrowth functions are necessary for mesosomal development in B. cereus 569 germination and outgrowth and it may be

possible to determine more of the function of these mesosomes by studying the apparent synthesis of extra membranes under A.D. treatment.

SECTION V

Production of extra membranes in *B. cereus* 569 by treatment with Actinomycin D: an autoradiographic study on exponential cells

SECTION V

Introduction

When germinated spores of B. cereus 569 are allowed to commence outgrowth and then treated with Actinomycin D (A.D.), membranous material appears to collect between the plasma membrane and new cell wall if the treatment is performed in a rich medium (Sect. IV). The extra membranes appear to originate from mesosomes, and are not produced in nutrient lack. The study also indicated that a period of RNA synthesis was required for the full development of mesosomes during outgrowth. However, the evidence for A.D.-resistant membrane synthesis was based entirely on electron-microscopy of thin sections and other interpretations could be made. It was possible in addition that any such effects were peculiar to germination and outgrowth. The effect of A.D. on exponential phase cells was therefore examined in detail, and subsequently cells were radioautographed with tritiated glycerol to establish whether extra membranes incorporated the label into their phospholipids during A.D. treatment. A parallel chemical analysis of residual phospholipid and protein synthesis during A.D. treatment was also performed (Sect. VI). The effect of A.D. on B. licheniformis 749c was also examined.

Materials and Methods

Organisms: Organisms used were B. cereus 569 and

B. licheniformis 749c; all exponential cultures were derived from spore stocks already described by loop inoculation into rich medium, and subcultured.

Media: Media used were L-broth (Sect. II), and L-broth supplemented with phosphate and magnesium (Sect. III).

Effect of A.D. on exponential cells: Four experiments were performed. The first established an effect on membranes, and the second investigated the time-course of the events observed in thin-section. The final two experiments investigated the effects in phosphate-magnesium supplemented medium, and whether a similar effect on membranes occurred in another organism, B. licheniformis 749c, under the same conditions.

a) A.D. effect on B. cereus 569 exponential cells. An exponential culture in L-broth was split into three equal portions; one portion was grown for 1 hour as a control, and another incubated for 1 hour with A.D. present. The third was incubated for 30 min. with A.D., and then spun down, washed three times and re-suspended in fresh L-broth and re-incubated for another 30 min. All three cultures were then immediately pre-fixed and fixed for electron-microscopy.

b) Time-course of A.D. effects. A.D. was added to an exponential culture of B. cereus 569 in L-broth. Samples for electron-microscopy were removed at 0, 2, 5, 10 and 20 min.

c) A.D. effect in supplemented L-broth. The

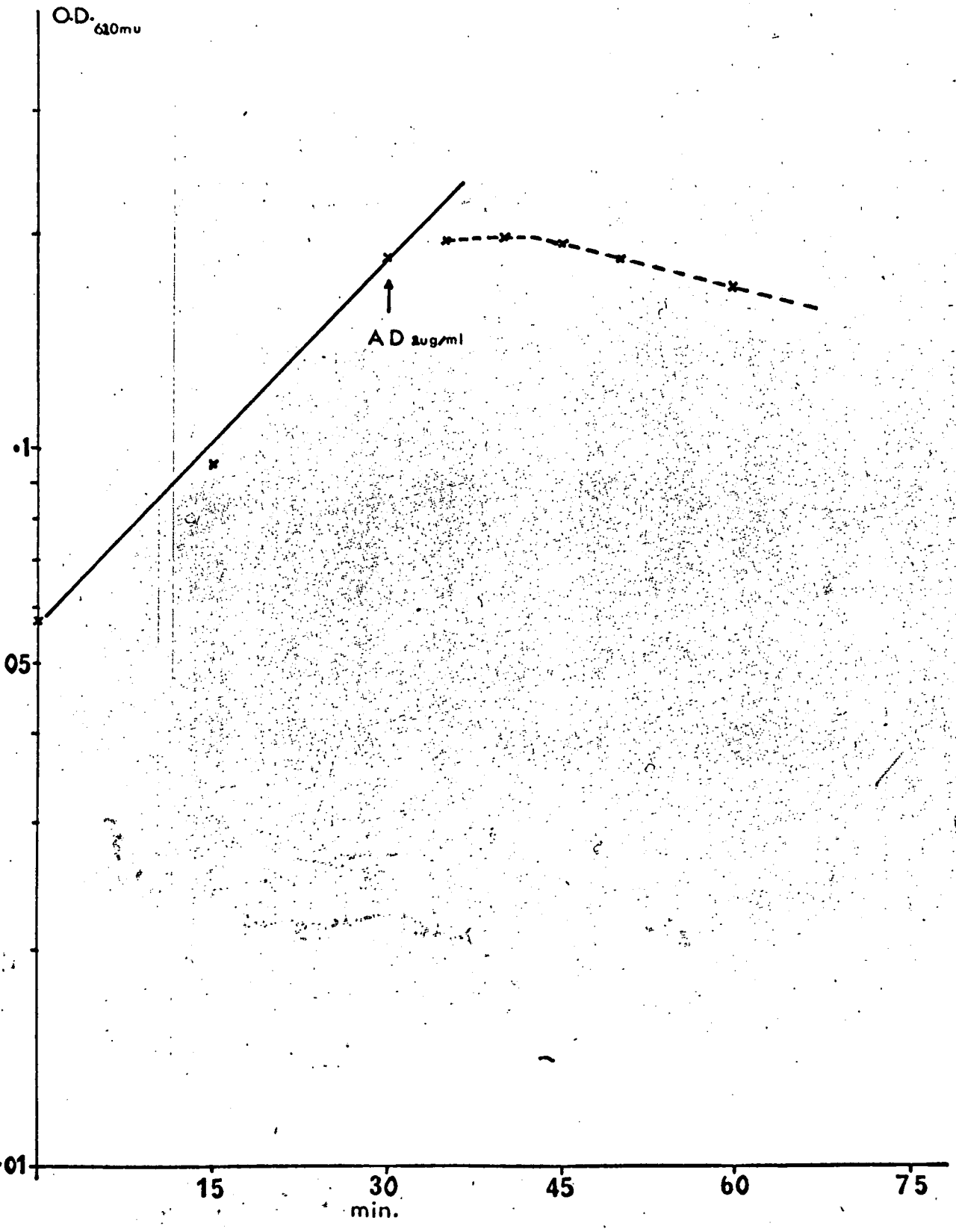
procedure given in a) was followed using supplemented L-broth.

d) A.D. effect on B. licheniformis 749c in L-broth. Procedure as for a).

Electron radioautography of glycerol-labelled cells: Three cultures of B. cereus 569 in L-broth were grown up into exponential phase. Two of them were inoculated with 2 ug/ml A.D., and after 1½ min. all three cultures were rapidly transferred to fresh flasks containing the equivalent of 50 uci/ml ~~tritiated~~ 2-^{3}H glycerol (final concentration) previously evaporated down in a gentle current of air to remove carrier alcohol, and re-incubated as follows: i) Control cells - incubated 15 min., cooled in ice, washed three times in fresh cold medium containing 2% "cold" glycerol, and reincubated a further 15 min. (pulse-chased culture); ii) the A.D. treated culture incubated 30 min.; iii) the remaining A.D. treated culture pulse-chased as for i), but using 30 min. incubation times. Each culture was then immediately pre-fixed and fixed. After overnight fixation, cultures ii) and iii) were washed three times in R-K buffer (Ryter, 1958) to remove native isotope in ii) and to act as a control in iii), and all specimens embedded in araldite as previously described. Specimens were sectioned with a diamond knife on a Huxley microtome at thicknesses producing silver-gold interference colours. Sections were picked up on 300 mesh nickel grids coated with a

FIG 1

Effect of A.D. on B.cereus 569 growth



collodion film, and post-stained with lead citrate. A thin film of carbon was then evaporated onto the grids.

Emulsion for radioautography was prepared by dissolving 10 g Ilford "L4" photographic emulsion in 25 ml distilled water at 40°C. After complete solution, the emulsion was left to gel for 15 min. in an ice-bucket. Grids for coating were fixed to slides by double-sided adhesive tape, 2 grids per slide, and the emulsion then applied using a 2-inch diameter fine-wire loop. Coated grids were dried for about 5 min., enclosed in light-tight boxes with desiccant, and left at 4°C for 6-7 weeks. Slides were developed in D126 developer for 2½ min. and fixed in freshly made thiosulphate fixative.

Cultures were all aerated and incubated at 37°C in flasks with a volume 5 x that of the culture.

Cell masses were measured by optical density (O.D.) at 620 mu in 10 mm path-length glass cells.

Electron microscopy was performed on a Siemens Elmiskop 1A operating at 80 KV at magnifications 10 - 20,000.

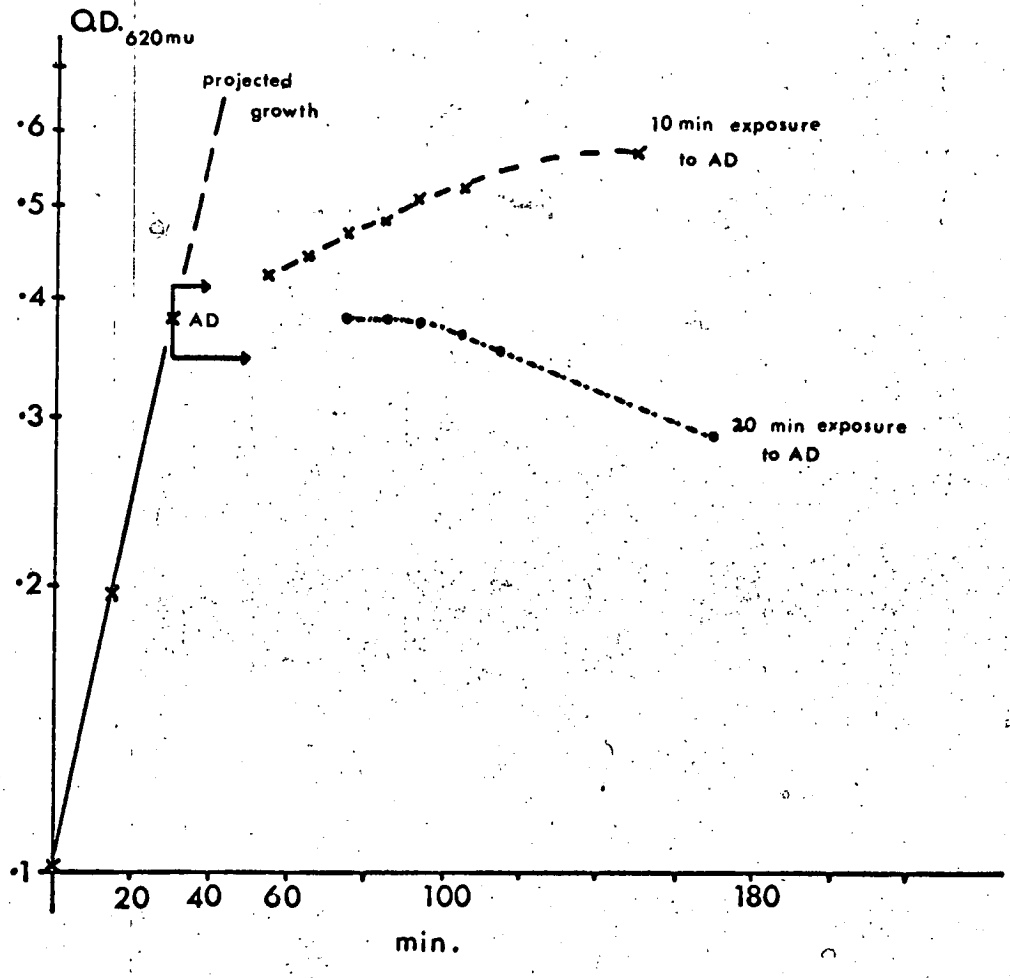
Results

a) Effect of A.D. on exponential B. cereus 569 cells

The effect of A.D. on exponential growth was investigated in the O.D. range 0.05 to 0.5, and at 2 ug/ml produced an almost immediate cessation of growth such as in Figure 1. In all experiments the residual O.D. increase after A.D. treatment was less than 10% of

FIG 2

Regrowth of B. cereus 569 after exposure to Actinomycin D (2µg/ml)



A culture was given A.D. at the point shown. After 10 min. one half was removed, washed three times and resuspended in fresh cold medium (L-broth) and then transferred to a clean pre-warmed flask. The second half of the culture was treated identically after 20 min. Incubations at 37°C, aerated by shaking.

normal after 15 minutes, and thereafter there was a gradual decline in O.D. presumably due to slow lysis. If cells were treated with A.D. for varying periods of time, and then washed and resuspended in fresh medium for further incubation, there was little recovery (Fig. 2). For electron microscopy A.D. was added at an O.D. of 0.4. The cultures treated with A.D. for 30 min. and 1 hour produced cells with extra membranes of the type found in outgrowing spores, lying between the wall and plasma-membranes (Plates 63, 64). Mesosomes were often present in areas of cells away from extra membranes. In both specimens the plasma membranes had 2 dark-staining layers, the DNA was somewhat broken up, and the distances between walls and plasma-membranes were generally increased relative to control cells. The amount of extra membrane appeared to be greater and more widespread in the culture subjected to a regrowth period of 30 min. after A.D., but this could have been accounted for by its decreased content of lysed and partially lysed cells; such cells, however, were infrequent in both. Extra membranes appeared to arise often from the vicinity of a mesosome and were frequently several layers thick; in some cells an extensive collection of vesicles was present. Serial section (Plates 65-71) showed that the space occupied by vesicles and lamellae could be very extensive.

An identical experiment performed at a higher O.D. (0.74) produced identical results (Plates 72, 73); data

were obtained from 50 randomly chosen longitudinal sections reproduced from negatives onto tracing-paper. Twenty-eight sections showed extra membrane lying between the wall and plasma-membrane somewhere in the section, and a further 9 sections showed lamellar mesosomes that could be judged increased in membrane content (cf. Sect. II). There was no preferential site for extra membrane to appear at. Ten cells had more than one mesosome that were probably not connected, but their size was often small in comparison to control cell mesosomes (cf. Sect. II, Plate 28).

b) Time-course of A.D. effect on B. cereus 569 cells

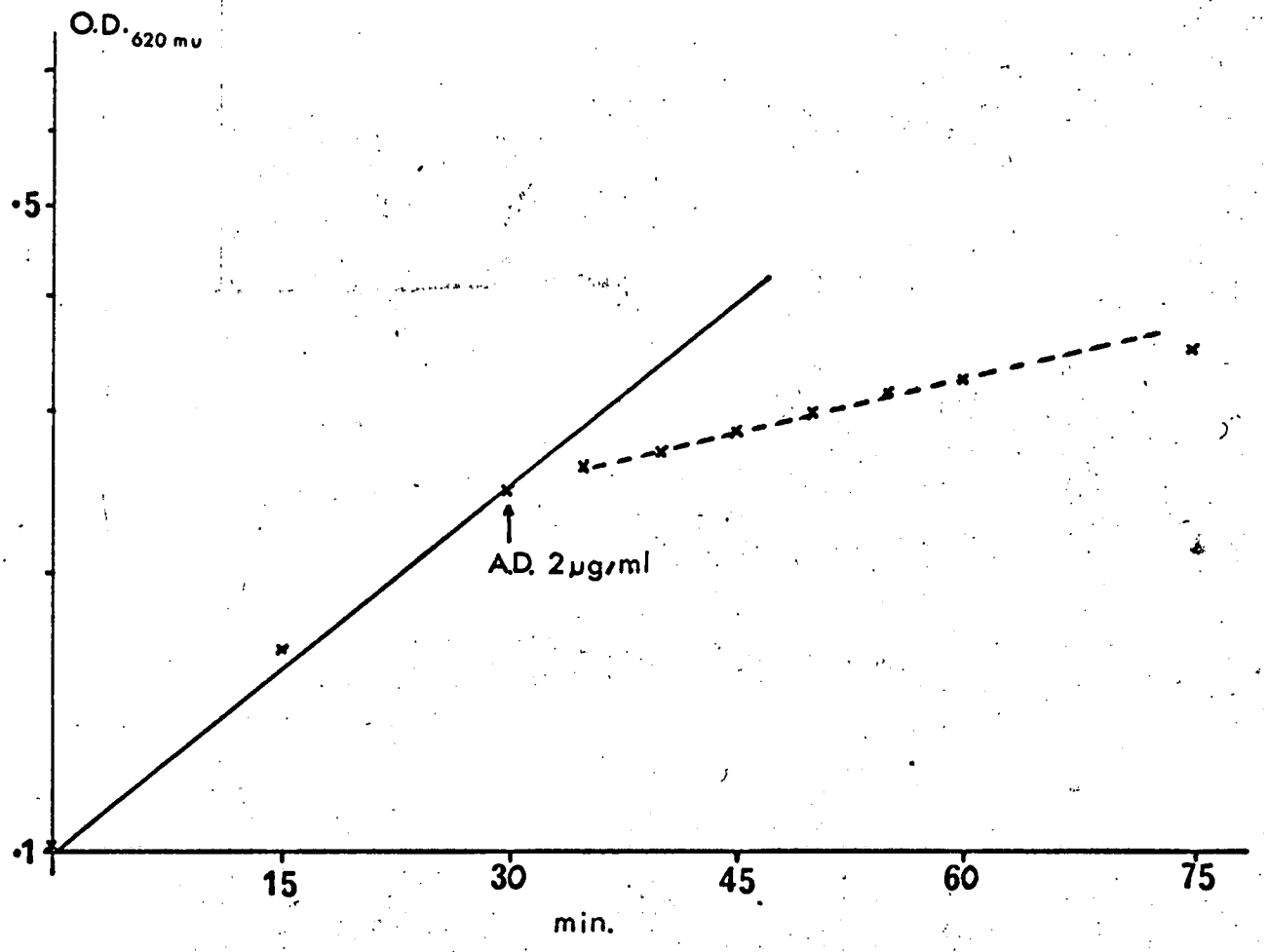
The experiment was commenced at an O.D. of 0.35. No significant changes were observed until the cells had been exposed to 20 min. A.D., apart from some compacting of the DNA. In the 20 min. sample, extra membrane appeared in about a fifth of all sections, but appeared to be less extensive in individual cells compared with previous 30 min. samples. The DNA was also taking on a more broken-up appearance.

c) Effect of A.D. on B. cereus 569 in supplemented L-broth

A.D. produced a similar effect on exponential growth in L-broth supplemented with phosphate and magnesium as in unsupplemented L-broth. Extra membrane was produced, but occurred in fewer sections (about 10%) where it appeared to be less extensive in comparison with extra membrane produced in cells treated for 30 or 60 min. with A.D. in unsupplemented L-broth.

FIG 3

Effect of A.D. on B. licheniformis 749c growth



The scale is the same as in Fig. 1.

d) Effect of A.D. on B. licheniformis 749c in L-broth

A.D. did not produce the rapid cessation of growth in B. licheniformis 749c culture observed with B. cereus 569, for, although there was an immediate effect, the O.D. continued to rise steadily (Fig. 3). In section, the cell walls were thickened and rather irregular, and the DNA was often concentrated in the long axis of the cell (Plate 74). Mesosomes were grossly disorganised, usually into rather small structures containing large vesicles. Only one case of extra membrane was observed, in association with a disorganised mesosome (Plate 75), and the mesosome disorganisation did not produce cells with more than one membranous area.

Electron radio-autography of glycerol-labelled cells

The development time was estimated from a simple calculation based on the activity of phospholipid extracted chemically (Sect. VI), and certain assumptions as follows: the specific activity of extractable phospholipid labelled during A.D. treatment was approximately 5×10^4 dpm for a total population of about 10^8 organisms, using label at an activity of 5 uci/ml. This is equivalent to 7×10^8 disintegrations per day at 50 uci/ml culture activity, and assuming that 10 sections are needed to serially section a cell in the long axis, and 10 disintegrations are required to produce one exposed grain, the average expected grain density would be about 1.4 per axial section after 20 days, exclusive of any

additional grains due to label elsewhere in the cell. A development time of 6 weeks was therefore chosen, and found to be suitable.

Examination of test grids showed satisfactory silver mono-layers in most areas (Plates 76, 77). In all experimental grids, background grains were low, usually about one per field, and areas were selected for photographic recording on the criteria of minimal emulsion reticulation and dirt, and good contrast in the underlying sections. Grains were classified as wall/membrane, mesosome or nucleus associated if any part of the grain was within 0.05 u (500 Å) of the respective structure. The commencing O.D. of each experiment was 0.5.

i) Control cells pulse-chased 15 min. each

The background count was negligible. Data were collected from 38 axial sections, redrawn directly from negatives. A total of 122 grains were counted, of which 28 were associated with nuclear material, 22 with mesosomes, and 37 with wall/membrane. Five grains could not be classified. However, these figures are liable to quite large error as a significant number of grains (2) overlapped 2 or more structures and classification was arbitrary. Plate 78 is a typical cell.

ii) Cells incubated 30 min. with A.D. and labelled glycerol

Grain counts were made on whole-field reproductions including part- and transverse-sections (Plate 79).

5173

5172

5171

5170

5169

5168

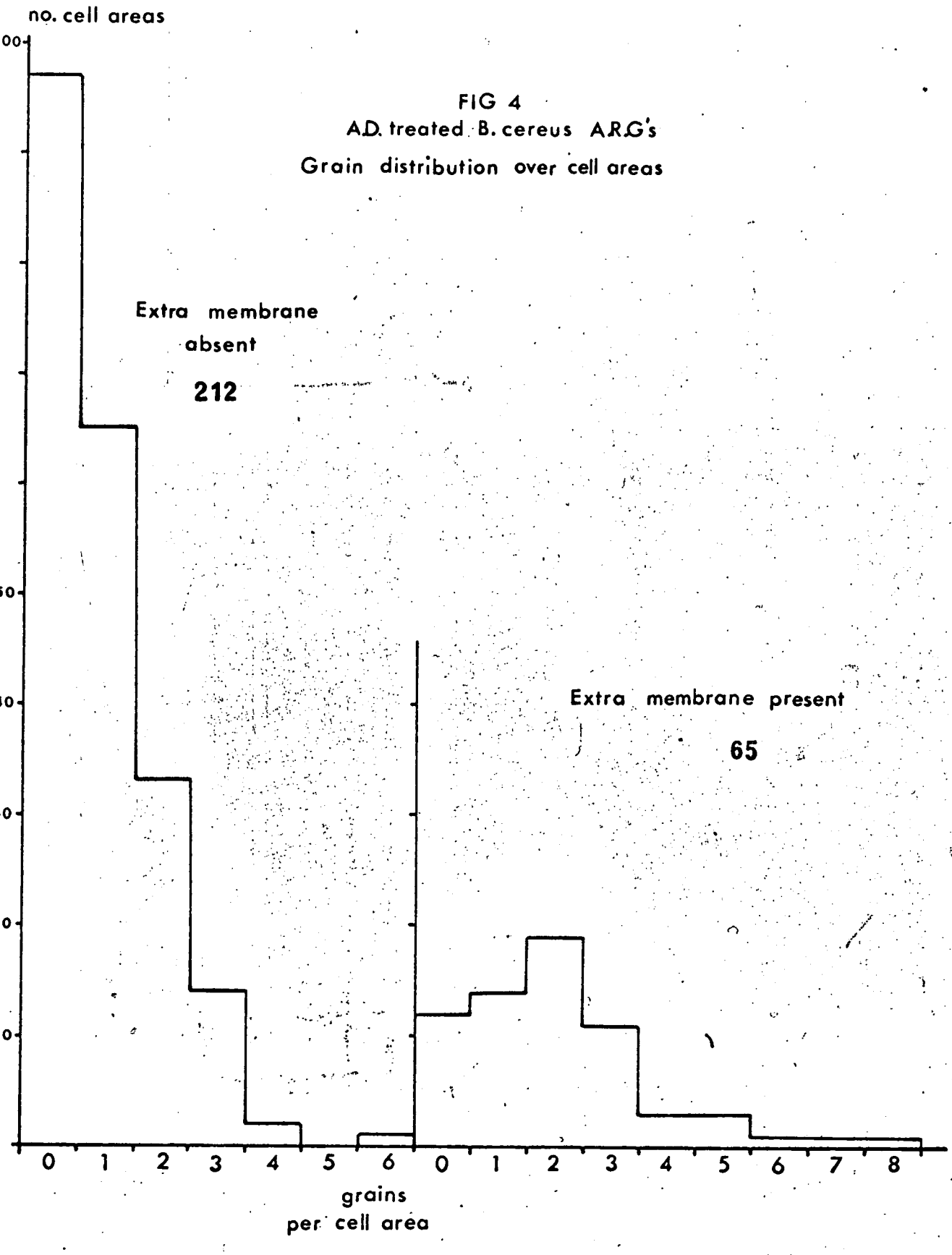


FIG 4
A.D. treated B. cereus A.R.G's
Grain distribution over cell areas

Figure 4 shows the distribution of 320 grains associated with wall or membranes (including mesosomes) over 277 cell areas. All other grains were ignored, and a cell area was defined as any section or part of section that contained a section of wall and/or membrane, thus a plane of wall was excluded. Both transverse and longitudinal sections were included and a correlation made with the frequency of extra membrane in authentic complete median transverse and longitudinal sections in the radio-autographic specimen and in a separate non-radio-autographic A.D. treated control. 35% and 37% of these complete median sections showed extra membrane somewhere in the section; in the grain survey, the frequency was 31%, thus establishing that the inclusion of part-sections did not materially distort the expected distribution of extra membranes. Figure 5 shows a reproduction of 25 cell areas; a total of 42 grains occur of which 8 are excluded (circled) as being non-localised. Eight cell areas occur with extra membranes present, and of the remaining 34 grains, 17 occur somewhere in these areas associated with the wall/membrane or extra membrane material. In the complete survey, the average grain count over cell areas not showing extra membrane was 0.87 per area; that for cell areas having extra membrane present somewhere was 2.1 per area.

iii) <u>Cells treated with A.D. and labelled, and then chased</u>

The appearances were closely similar to those above. A full survey was not undertaken, but a small sample

produced a grain distribution indicating that there were no significant differences.

Discussion

The production of extra membranes on treatment with A.D. appears to be the normal response in B. cereus 569 cells. A number of explanations are possible, and it was necessary to decide whether extra membrane production was due to new synthesis, or some form of membrane re-distribution without new synthesis. Two factors in favour of the latter are that mesosomes are easily everted (Highton, 1969), and on treatment with A.D. mesosomes elsewhere in the cell appear to be reduced in size, and possibly in number. An estimate can be made, however, on the amounts of membrane material in mesosomes of a normal cell and in the extra membranes found in A.D. treated cells. Thus, taking a typical cell of 1.7 u long and 0.65 u in diameter and assuming hemispherical ends, the total surface area of plasma membrane is about 4.5 u^2, of which about 0.6 u^2 covers each hemispherical end. A mesosome of 0.2 u diameter containing 5 concentric lamellae spaced at about 0.02 u apart would have a total membranous area of just over 0.2 u^2, and three of such mesosomes would have a total membranous area of about 0.7 u^2. In such a cell there is therefore enough total mesosome membrane to produce one extra membrane round one end of the cell as a hemispherical sheet. Such a calculation is of course liable to fairly gross

error depending on the cell size and mesosome structure and number, but in a significant number of cells it is clear that the amount of extra membrane, exclusive of any other mesosomes present, is greatly in excess of what might reasonably be expected if all the mesosomes had been reorganised into extra membrane (Plates 65-71). Evidence further supporting synthesis of new membrane is that new phospholipids are synthesised during A.D. inhibition, as shown by uptake of labelled glycerol into phospholipid fractions (Sect. VI), and that when such cells are radio-autographed, there are more grains on average over sections with extra membrane present than over sections where extra membrane is absent, although it cannot be concluded that the extra grains arise from the extra membranes, due to the low resolution of the technique and the inability to estimate with any reliability the average amount of extra membrane contained in a section. The origin of grains in sections not containing extra membrane is probably wall teichoic acid, assuming the plasma membrane does not turn over to any extent; whether the apparent reduction in extra membranes when supplemented L-broth is used indicates their involvement in teichoic acid synthesis, which appears to be partly dependent on phosphate and magnesium concentrations (Ellwood, 1970), is very uncertain. However, the observation that extra membranes are produced only after a time-lag of about 15 min. is more compatible with the membranes being newly synthesised

than gradually reorganised.

Almost nothing is known of the control of mesosome membrane synthesis. Studies with a glycerol auxotroph of B. subtilis showed that proteins were incorporated into unspecified membrane fractions in the absence of lipid synthesis (Mindich, 1970) although glycerol-starved cells had an apparently normal thin-section appearance. The production of extra membranes in Streptomyces olivaceous by treatment with Mitomycin C has been reported (Mach, 1964) but the origin of the membranous material was most unclear and could not be related to mesosomes known to be present in other streptomyces species (Hopwood, 1960) from the photomicrographs presented. The previous studies have shown that there is a different mesosome arrangement in B. cereus 569 from B. licheniformis and imply that their method of replication or growth in dividing cells is also different; thus, those in B. licheniformis divide with the cells whilst those in B. cereus are constantly synthesised in new positions in the cell. This implication is strongly supported by the finding that the response of the membranes in A.D. treatment is different in the two species. In part, this may be due to a significant residual cell growth in A.D. treated B. licheniformis (Fig. 3), but this in itself raises the question as to why the inhibition in B. cereus is so immediate (Fig. 1). The effect of A.D. is primarily to inhibit RNA synthesis (Sect. IV), and it would appear that cell growth in B. cereus 569 may

therefore be tightly coupled to some very short-lived mRNA. If this is so, however, the relatively poor inhibition of growth by chloramphenicol is difficult to understand if RNAs only act by producing protein.

It has not been possible to recognise any particular pattern in the site of production of extra membranes, which might help to clarify mesosome function or behaviour. Numerous chemical studies have been made on membrane fractions released by lysosyme, for example, by Reavely (1969) and Ferrandes (1966); this method, however, leaves considerable uncertainty of the origin of the membrane fractions analysed. Whilst it is doubtful also that extra membranes produced in A.D. treatment are "normal" mesosome membranes, the possibility now clearly exists for recognising different mesosome systems with different controls for synthesis and function, and the investigation of aberrant mesosome membranes may supply more conclusive evidence on their function <u>in vivo</u>.

SECTION VI

Lipids of *B. cereus* 569 and their synthesis during treatment with Actinomycin D

SECTION VI

Introduction

Thin-section appearances of B. cereus 569 cells treated with Actinomycin D (A.D.) suggested that the synthesis of membranes, possibly of mesosomal origin, continued whilst cell growth was arrested. A study was therefore performed on the nature of normal B. cereus 569 cell lipids and of their synthesis during A.D. treatment using labelled lipid precursor (tritiated glycerol).

Materials and Methods

Organism: B. cereus 569 cultures were grown up from the spore stocks already described.

Media: Media used were L-broth, and L-broth supplemented with phosphate and magnesium.

Lipid extraction and procedures: Twelve litre cultures were grown up in a "Microferm" (New Brunswick Scientific Co.) laboratory fermenter aerated at 37°C, and harvested at optical densities about 0.4 at 620 mu by centrifugation. The resulting cell paste was washed three times in 0.05 M phosphate buffer pH 7 containing 10^{-2} M magnesium, followed by a 0.85% saline wash, and the cells freeze-dried. A known weight of dry cells was then extracted overnight at room temperature with 50 volumes of chloroform-methanol 2:1 (C-M). The extract was then evaporated down on a rotary evaporator and resuspended in a small quantity of C-M 2:1 for

purification. Two methods were used for lipid purification: that of Wuthier (1966) using the 2-phase system of Folch (see Wuthier, 1966); and a simpler purification used subsequently. In the first method, Sephadex E25 was equilibrated with Folch upper-phase and packed into a 9.6 x 1 cm column. The column was washed with 1 void volume of Folch lower-phase, and the lipid applied to the column in a small quantity (about 2 ml) of lower-phase after evaporating down the crude lipid extract. The column was then run at 0.2 ml/min. with 25 ml lower-phase, and the purified eluate evaporated to dryness. Alternatively, Sephadex E25 was equilibrated with chloroform-methanol-water 65:25:4 (CMW), and the purification performed using this one-phase system as described for Wuthier's method.

Chromatography: Purified lipid was chromatogrammed on 1 mm thin layer silica-gel plates previously oven-dried. Solvents used were CMW 65:25:4, and C-M-acetic acid-W 80:13:8:0.3. Spots were detected by spraying with 2% ninhydrin in butanol for amino groups, Periodate-Schiff's reagent for free vicinal hydroxyl groups and sulphuric molybdate (Skipski, 1969) for phosphate groups. Total lipids were identified by charring at 100°C after spraying with molybdate reagent.

Deacylation: Approximately 10 mg of lipid was dissolved in 4.5 ml CM 1:1. An equal volume of sodium methoxide was added, and the reaction mixture left at room temperature for 30 min. Five ml water was then

added, and the mixture neutralised by adding 50 W x 12 Dowex ion-exchange resin (H^+ form). The aqueous phase was separated from the chloroform phase by filtration through Whatman 1PS silicone-treated filter paper, and both phases evaporated to dryness. The fatty acid methyl esters (chloroform phase) were dissolved in a small quantity of ether and examined by gas chromatography on DECS at 160°C using Argon carrier at a flow rate of 35 ml/min. Authentic standards were used for comparison. The phosphate esters (aqueous phase) were redissolved in a small quantity of water and subjected to descending paper chromatography (Whatman No. 1) in propanol-ammonia (880 SG)-water 6:3:1 (PNW).

Acid hydrolysis: Deacylated lipid (aqueous phase above) was subjected to hydrolysis in 1 N HCl for varying times (1-6 hours) at 100°C. After neutralisation with ion exchange resin (OH^- form) the products were chromatogrammed (descending) in PNW and butanal-pyridine-water 6:4:3 (BPW) using Whatman No. 1 paper.

Spots separated by paper chromatography were identified with ninhydrin, Periodate-Schiff reagent, Haines-Iscerwood (Haines, 1949) reagent for phosphate using UV light as a developer, and alkaline silver-nitrate for reducing sugars.

Standards were prepared from samples of phosphatidyle ethanolamine (PE), lyso-PE and cardiolipin (CL) supplied by Koch-Light laboratories.

Uptake of labelled glycerol: C-2 tritiated glycerol

(Radiochemical Centre, Amersham) S.A. 100 mci/mM was used to study the rate and amount of new lipid synthesis during A.D. treatment. For total uptake studies, small (10-25 ml) cultures in L-broth and supplemented L-broth were inoculated with 2 ug/ml A.D. Two minutes after this addition, tritiated glycerol (G2T) was added to a final activity of 1-5 uci/ml and the incubation continued for 30 min. The cultures were then immediately cooled and $\frac{1}{50}$th volume of 20% "cold" glycerol added to prevent further incorporation. After a series of 3 washes with 0.05 M phosphate buffer containing 10^{-2} M magnesium, the cells were extracted overnight at room temperature with 50 ml CM 2:1. After evaporation and resuspension in a small volume (1-2 ml) CM 2:1, the extract was purified on small (6 x 0.5 cm) sephadex columns by the methods described. Purified lipid was evaporated and resuspended in a known volume of CM 2:1, usually 1 ml, and known volumes of usually 0.1 ml counted in Dioxan scintillant.

The nature of the radioactivity isolated in the lipid fractions was investigated by silica gel thin-layer chromatography in CMW 65:25:4. For this, the experiments were conducted as described above but with culture activities of 50 uci/ml using tracer from which alcohol carrier had been removed by air evaporation, and therefore requiring transfer of cultures to the pre-warmed flasks containing G2T. After the chromatograms had been run, they were allowed to dry thoroughly at room temperature; the gel was then divided up into 1 cm strips and scraped

off the plates into vials containing 5 ml Dioxan scintillant for counting.

Radioactivity in the cell residue after lipid extraction was measured after hydrolysis for 4 hours in 1 N HCl at 100°C followed by desiccation over sodium hydroxide; the hydrolysed residue was redissolved in a known small quantity of water, and 0.1 ml amounts counted in Dioxan.

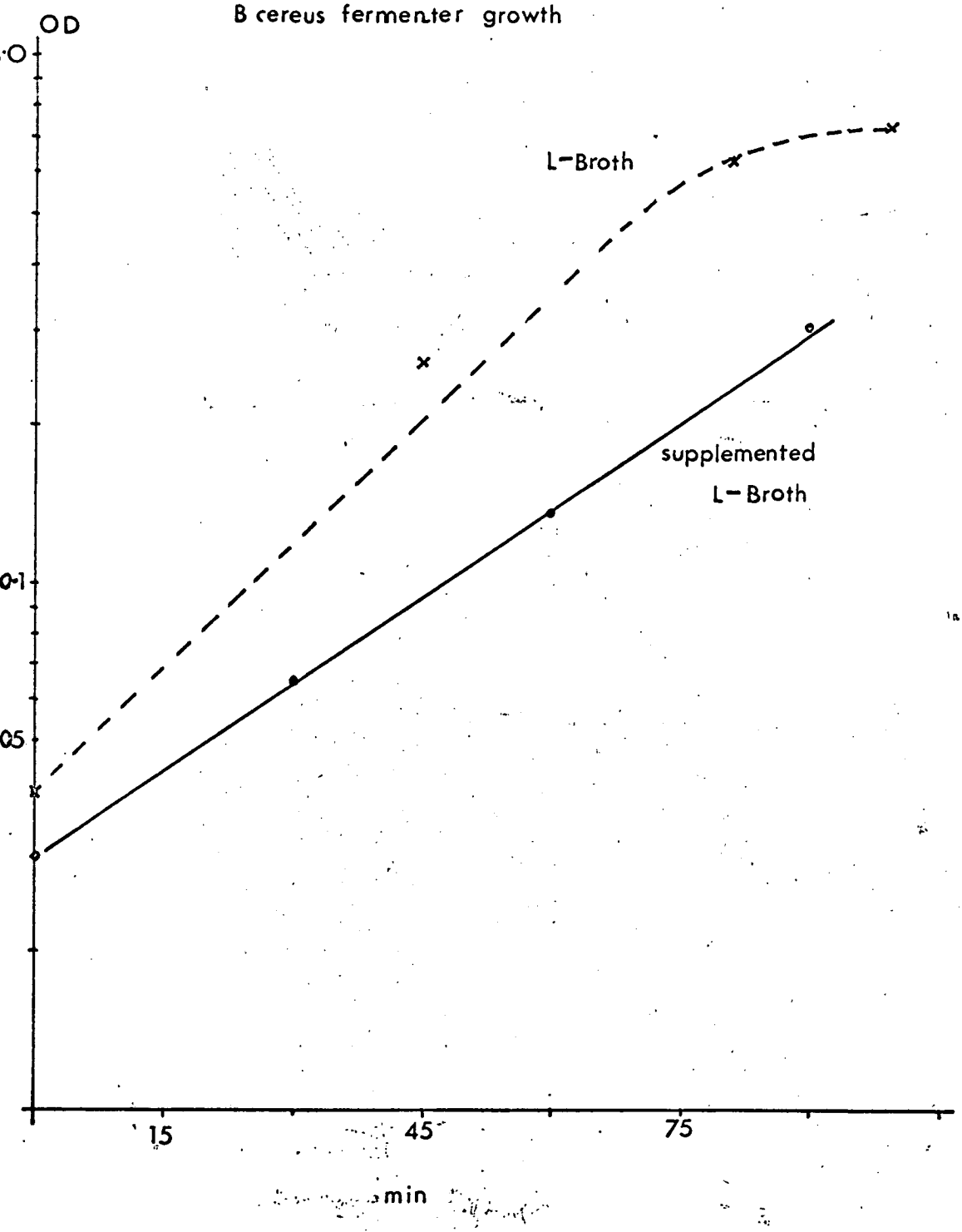
The kinetics of G2T uptake was followed by removing known volumes of A.D. treated, G2T labelled cultures at various time intervals, and measuring the incorporation into extracted lipid by the methods described above.

Uptake of ^{14}C leucine: Residual protein synthesis during A.D. treatment was followed by measuring the rate of accumulation of ^{14}C labelled L-leucine in TCA precipitates. Fifty ml cultures in L-broth and supplemented L-broth were inoculated with A.D. 2 ug/ml, and received 0.28 uci/ml ^{14}C leucine (270 mci/uM). At various times, 10 ml volumes were removed and pipetted into ice-cooled tubes containing 1 ml 50% TCA and 0.2 ml of 1% "cold" leucine carrier. After 1-1½ hours at 4°C, the entire contents of each tube were washed onto glass-fibre filters, washed 3 times with cold 5% TCA, 2 times with ether, and 2 times with absolute alcohol and dried in air. Filters were counted in 5 ml Dioxan scintillant.

Total phosphate was estimated by molybdenum-ascorbic acid (Ames, 1966) after washing with 75% perchloric acid at 100° until a clear colourless solution remained.

FIG 1

B cereus fermenter growth



Scintillant for radioactive counting contained naphthalene 130 g, PPO 8 g and dimethyl POPOP 0.3 g per litre of Dioxan, and cell counts were performed on a Beckman scintillation counter. Unless otherwise stated, all results are given as background-subtracted and corrected for quenching on the basis of quench correction curves constructed with the same isotope.

Electron microscopy was performed by the methods previously described.

Cell masses were measured by optical density (O.D.) at 620 mu using 10 mm path-length glass cells.

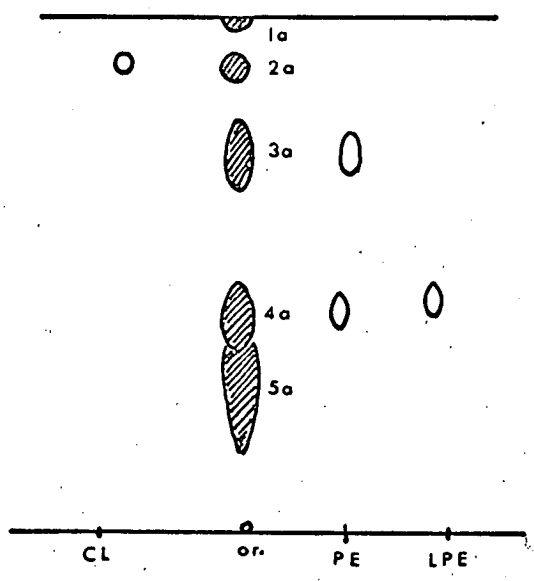
Results

Lipids of B. cereus 569 grown in L-broth

The mass doubling-times of the fermenter cultures in both L-broth and supplemented L-broth were somewhat variable, but were usually between 20 and 30 min. (Fig. 1). Cells were harvested between O.D.s 0.4 and 0.7 in both media, and were considered to be in the late exponential phase.

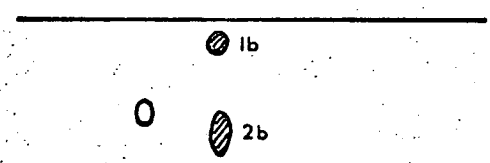
1.5 mg lipid phosphorus were obtained after extraction, purification and digestion from 2.4 g dry weight of cells. Applying a conversion factor of 32, this is equivalent to approximately 19 mg phospho-lipid/g dry weight of cells (2%). Figure 2 shows the order of major spots separated by thin-layer chromatography (TLC) in the two solvent systems, a and b. R_f values are not given, as these were not reproducible to a useful extent,

FIG 2



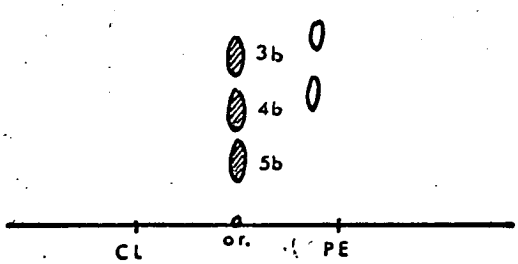
A

CMW 65-25-4



B

CMHAcW 80-13-8-03



most likely because of the extreme volatility of some of the solvent components, and variations in gel thickness and hydration. Table 1 shows the reactions of the various spots:

Component	Solvent	Ninhydrin	Phospho-molybdate	Schiff	Charring
1a	CMW	-	-	-	+
2a		-	+	+	+
3a		+	+	(+)	+
4a		+	+	(+)	+
5a		-	+	++	+
1b	CMHAcW	-	-	-	+
2b		-	+	+	+
3b		+	+	(+)	+
4b		+	+	(+)	+
5b		-	+	++	+

The reactions of the standards were as follows (see Fig. 2):

a) Cardiolipin: One component, phosphomolybdate +, Schiff +, ninhydrin -ve. Runs with 2a and 2b.

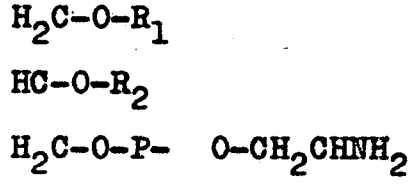
b) Phosphatidyle ethanolamine: Two components, in both systems. Both components reactive to all reagents, but reduce Schiff base slowly. First runs with 3a and 3b, second with 4a and 4b and lyso-PE.

c) Lyso-PE: One component, phosphomolybdate +, ninhydrin +, Schiff -ve. Runs with 4a, 4b and the

Structural Formulae of Phospholipids

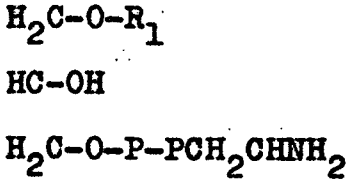
FIGURE 3

a) Phosphatidyle ethanolamine (PE)

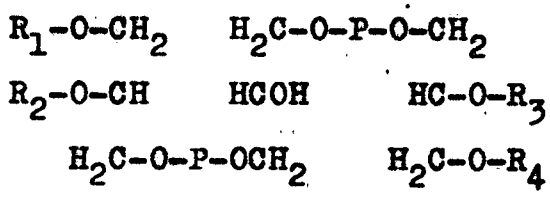


ethanolamine

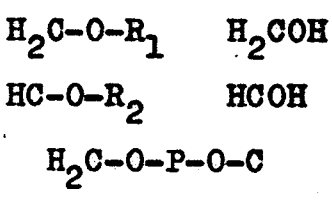
b) Lyso PE



c) Cardiolipin (CL)

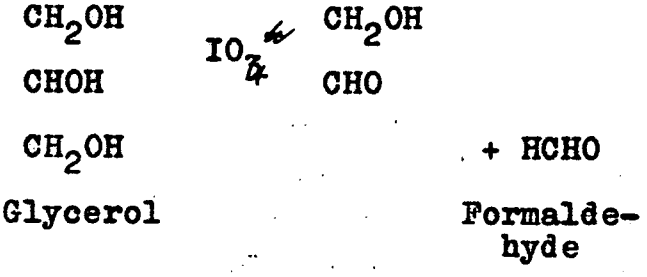


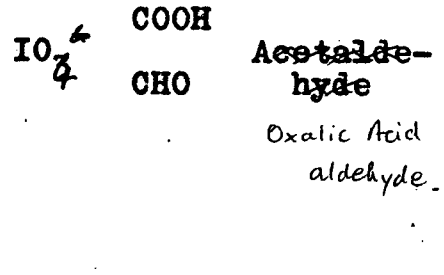
d) Phosphatidyl glycerol (PG)



R_1, R_2... are ester-linked fatty acids.

In the mechanism of the Periodate Schiff reaction, adjacent hydroxyls are oxidised firstly by periodic acid, releasing formaldehyde and the corresponding aldehyde; for example, in glycerol:





The formaldehyde released reacts with colourless Schiff reagent ~~base (p. Rosaniline~~) to produce a violet colour. Thus, only Pc~~to~~ above will be reactive, unless fatty acids are removed (deacylation) to expose vicinal hydroxyls.

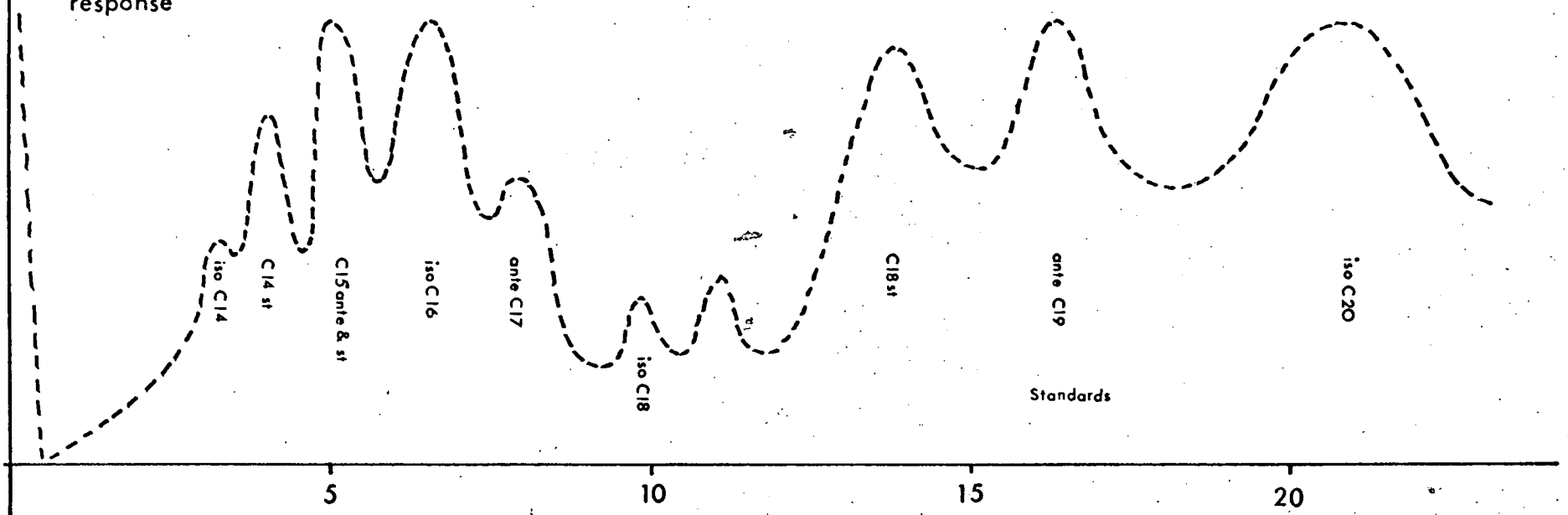

response
iso C14
C14 st
C15 ante & st
iso C16
ante C17
iso C18
C18 st
ante C19
iso C20
Standards
5
10
15
20
cms.

FIG 4

second component of PE above.

On the basis of their mobilities in relation to the standards, and their reactions above, the spots were identified as follows:

1a and 1b are neutral lipid, and are not adsorbed to the gel, thus being carried with the solvent front.

2a and 2b are cardiolipin (CL).

3a and 3b are phosphatidylϕ ethanolamine (PE).

4a and 4b are lyso-PE.

5a and 5b are phosphatidylϕ glycerol (PG), reacting strongly after oxidation with Schiff reagent (Table 2) on account of the free hydroxyls of the glycerol moiety. Structural formulae of these lipids are given in Figure 3. The reaction of PE and CL with Schiff reagent is not understood, but may relate to partial deacylation as obviously had occurred in the PE standard.

Deacylation products

a) Fatty-acid methyl esters: Figure 4 shows the peaks recorded on gas-chromatography; the following fatty acids were provisionally identified, but the study was not proceeded with further:

1. C-14 straight-chain.
2. iso-C 16 branched-chain.
3. ante-iso C 17 branched-chain.
4. iso-C 18 branched-chain.
5. ante-iso C 19 branched-chain.

b) Phosphate esters: Table 2 shows the reactions of spots separated by paper chromatography (Whatman No. 1 paper) in PNW. Values are given as $R_{glycerol}$ ($R_{glycerol}$ = 1).

Table 2

$R_{glycerol}$	Ninhydrin	Molybdate	Schiff	Identification	Origin
0.38	-	+	-	? Glycerophosphate	?
0.58	+	(+)	(-)	GPE	PE
0.63	-	+	+	GPGPG	CL
0.71	-	+	+	GPG	PG

The identity of the first component is not certain; it ran with ∝GP standard but did not react to Schiff's reagent. Glycerophosphorylethanolamine (GPE) was not clearly separated from diphosphatidylglycerol (GPGPG), and reacted poorly to Schiff's reagent. A small amount of glucose was detected by silver nitrate after paper chromatography in BPW, possibly from undetected glycolipid. Acid hydrolysis of deacylated lipid with 1 N HCl for 6 hrs produced 3 components after paper chromatography in PNW, corresponding in mobilities and colour reactions to inorganic phosphate (at origin), ∝GP ($R_{glycerol}$ 0.38) and ethanolamine, together with varying amounts of glycerol.

Lipids of B. cereus 569 grown in supplemented L-broth

19.2 mg phospholipid were extracted from 1 g dry weight of cells. There were no essential differences on

TLC of purified lipid, After deacylation and chromatography in PNW using Whatman No. 1 paper, 4 water-soluble phosphate esters were separated (Table 3):

Table 3

$R_{glycerol}$	Ninhydrin	Molybdate	Schiff	Identification	Origin
0.38	-	+	+	GP	?
0.69	-	+	+	GPGPG	CL
0.72	+	+	(-)	GPE	PE
0.81	-	+	+	GPG	PG

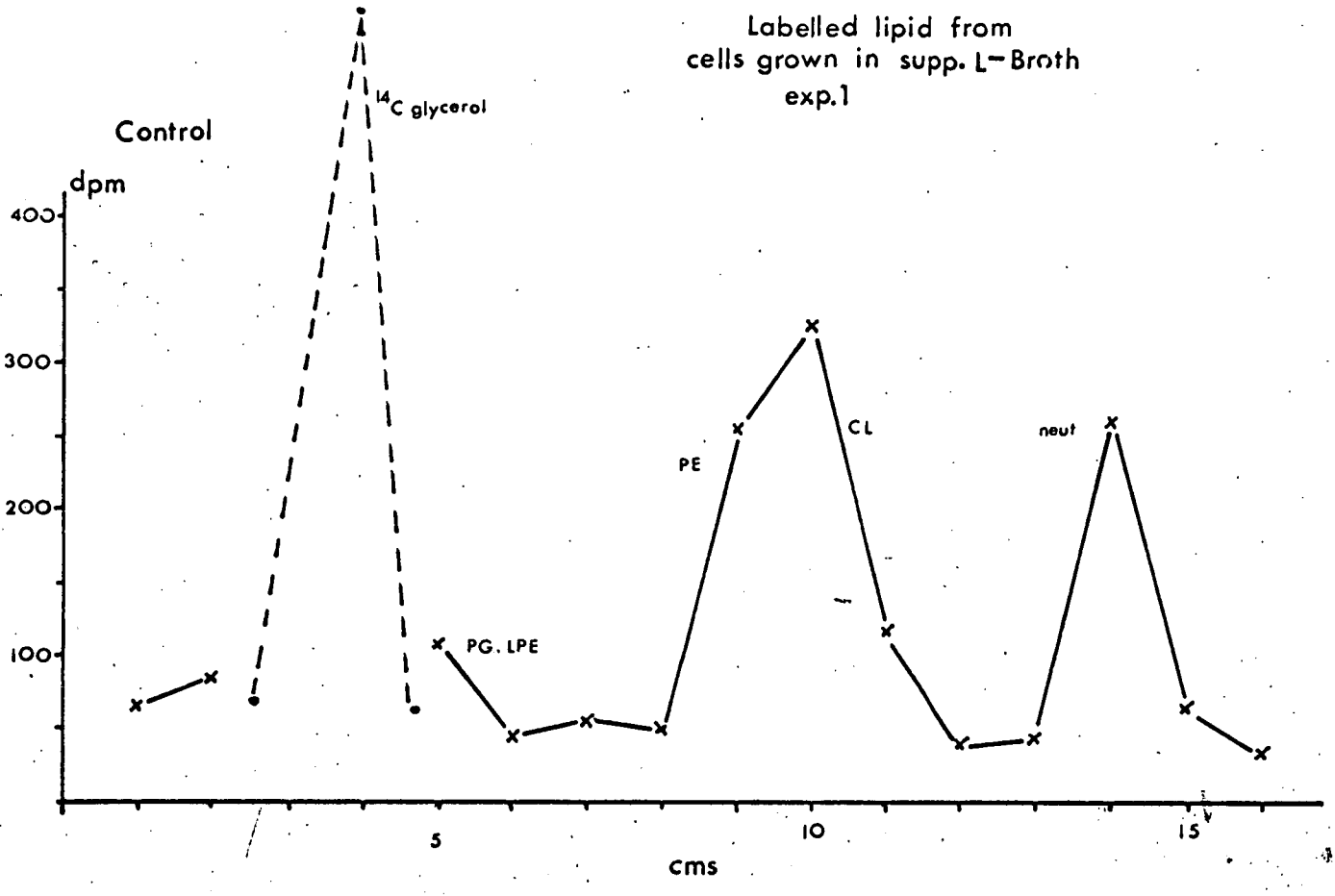
Acid hydrolysis of deacylated lipid produced inorganic phosphate, GP, ethanolamine and glycerol, as identified by mobilities and colour reactions. The fatty acids were not investigated, and no sugars were detected.

Incorporation of G2T during A.D. treatment

The addition of G2T had no effect either on the normal growth rate or on A.D. inhibition at the concentration used; previous electron-microscope experiments, however, had shown that extra membrane was inhibited by alcohol carrier at concentrations above 5%, hence alcohol was removed by evaporation when using high levels of isotope. Counts performed on wash supernates showed a reduction factor of at least 10^2 in native isotope activity at each wash. In both media, significant activity was present in the lipid fractions after 30 min.

FIG 7

Labelled lipid from cells grown in supp. L-Broth exp.1



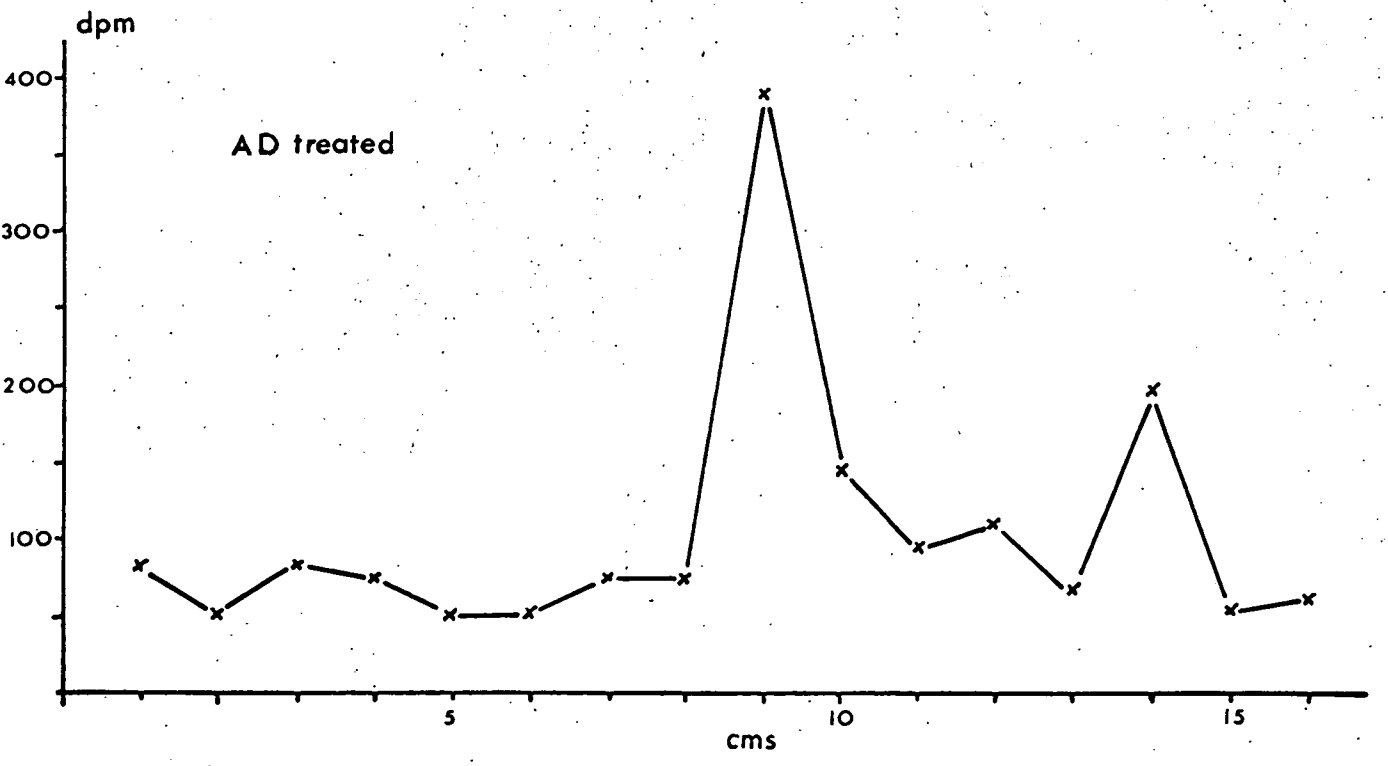
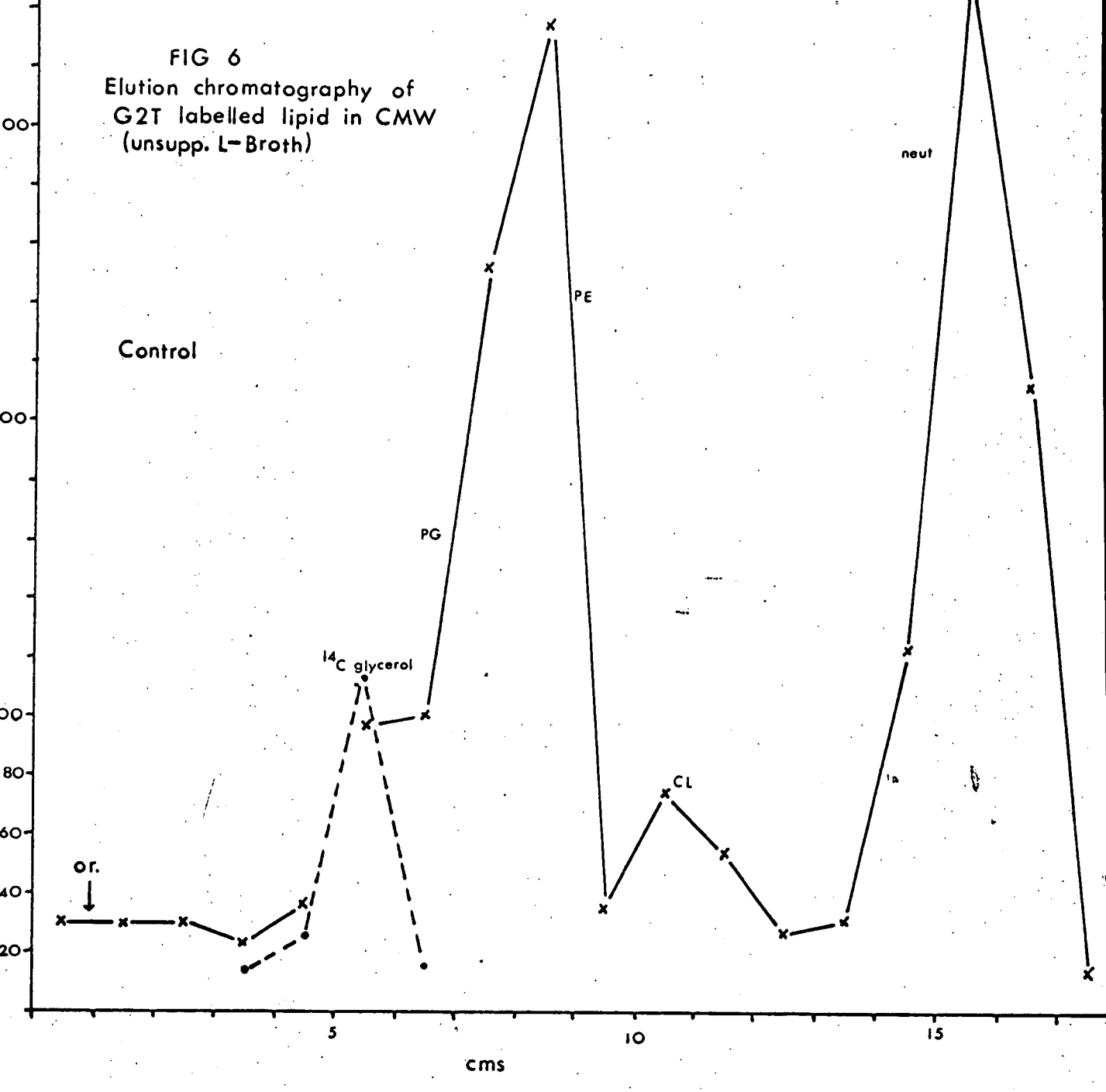


FIG 6
Elution chromatography of
G2T labelled lipid in CMW
(unsupp. L-Broth)



AD treated

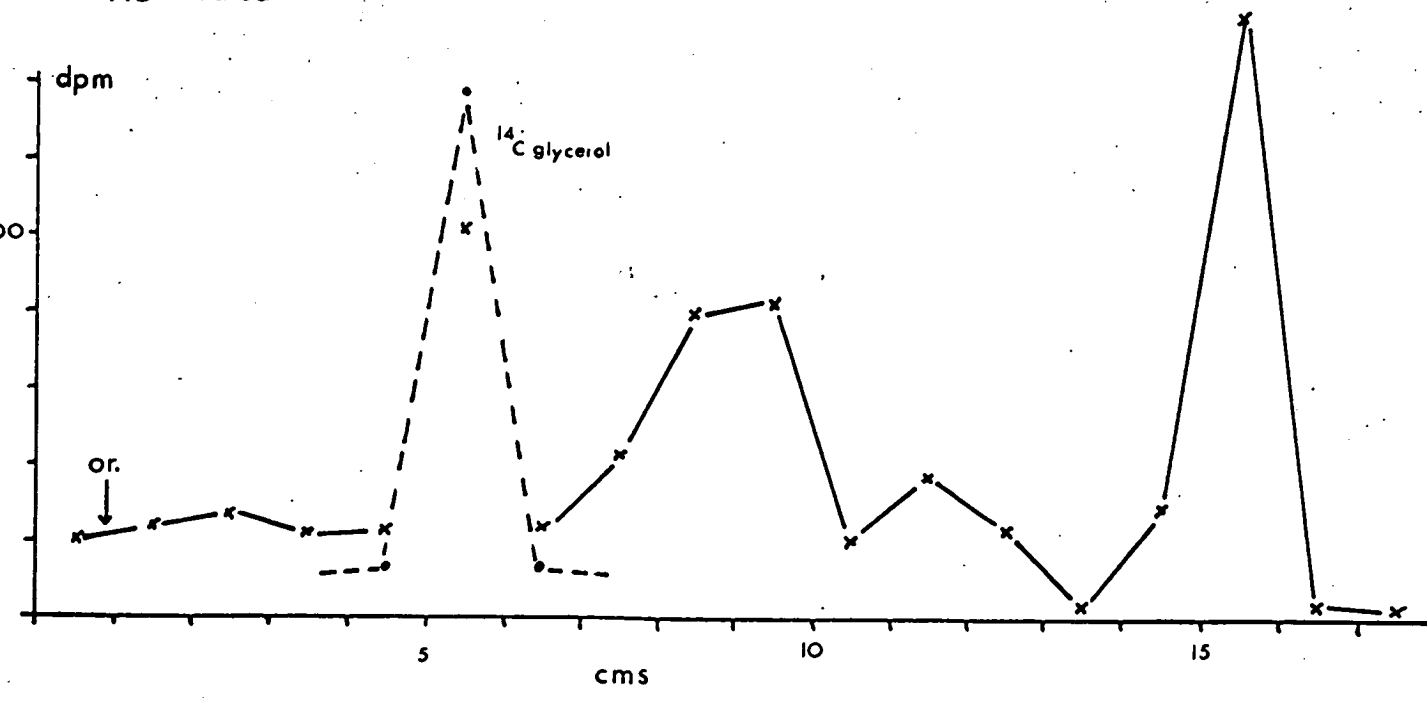
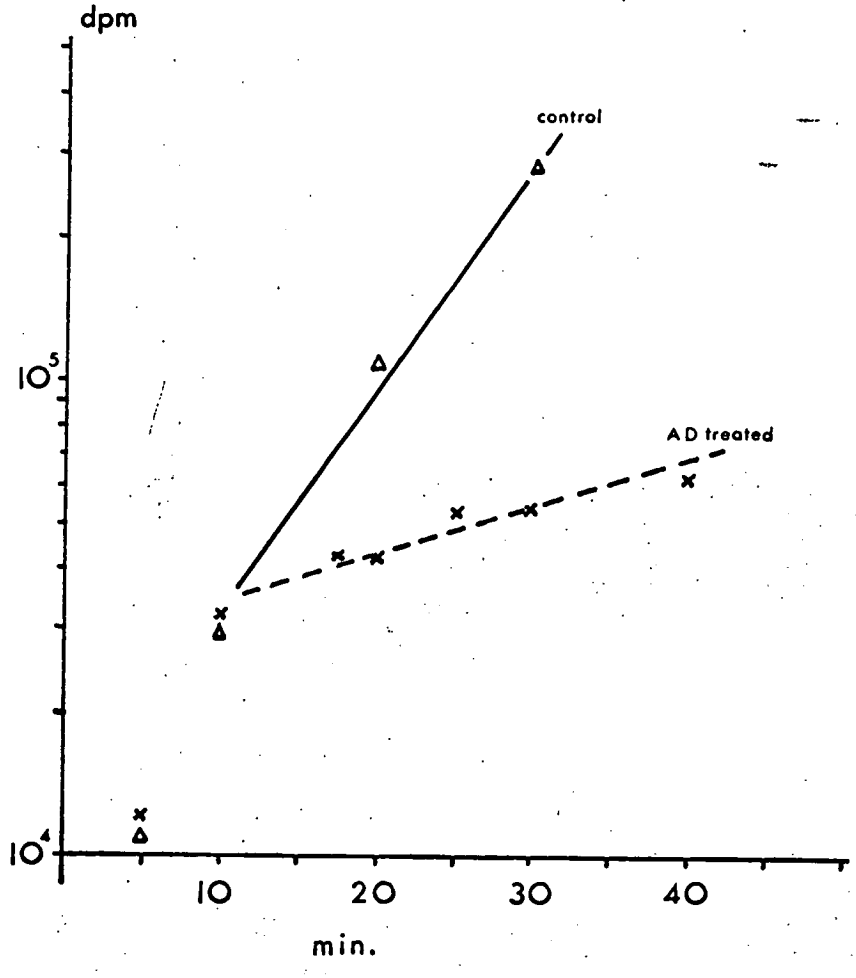


FIG 5

Incorporation of G2T (5uci/ml) into total lipid



exposure to A.D. and G2T (Table 4). (Activities for A.D. treated cultures are corrected to an O.D. 0.6 at isotope concentration 5 uci/ml; all counts are total extractable activity.)

Table 4

Medium	Control cell lipid	O.D.s		A.D. cell lipid (O.D.s static)
L-broth	280,000	0.42	0.62	62,000
Supplemented L-broth	390,000	0.47	1.55	40,000

Table 5 shows the distribution of label in lipid and residue fractions of A.D. treated cells:

Table 5

Medium	Lipid	Residue	% counts in lipid
L-broth	62,000	250,000	20
Supplemented L-broth	40,000	46,000	46

The kinetics of the lipid labelling (Fig. 5) show that activity in the lipid fraction continues to increase during the whole of the labelling period with A.D. present, but at a much lower rate than for control cells.

Nature of labelled lipid

Labelled lipid was subjected to thin-layer chromatography in CMW, and elution chromatograms obtained of the activity (Figs. 6, 7). Activity was clearly separable from native isotope (^{14}C glycerol marker) and lipid labelled during A.D. treatment had radioactive

FIG 9

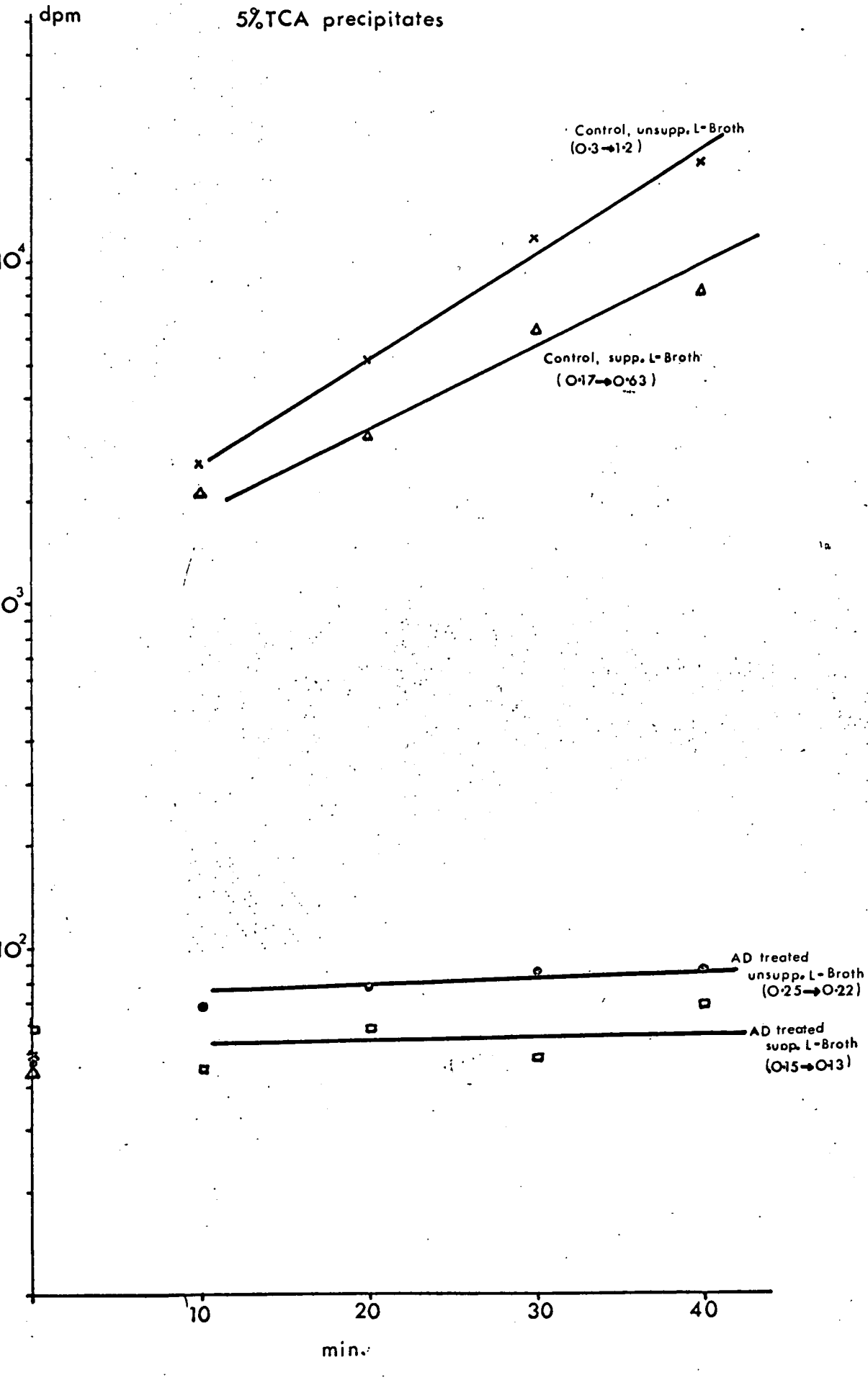
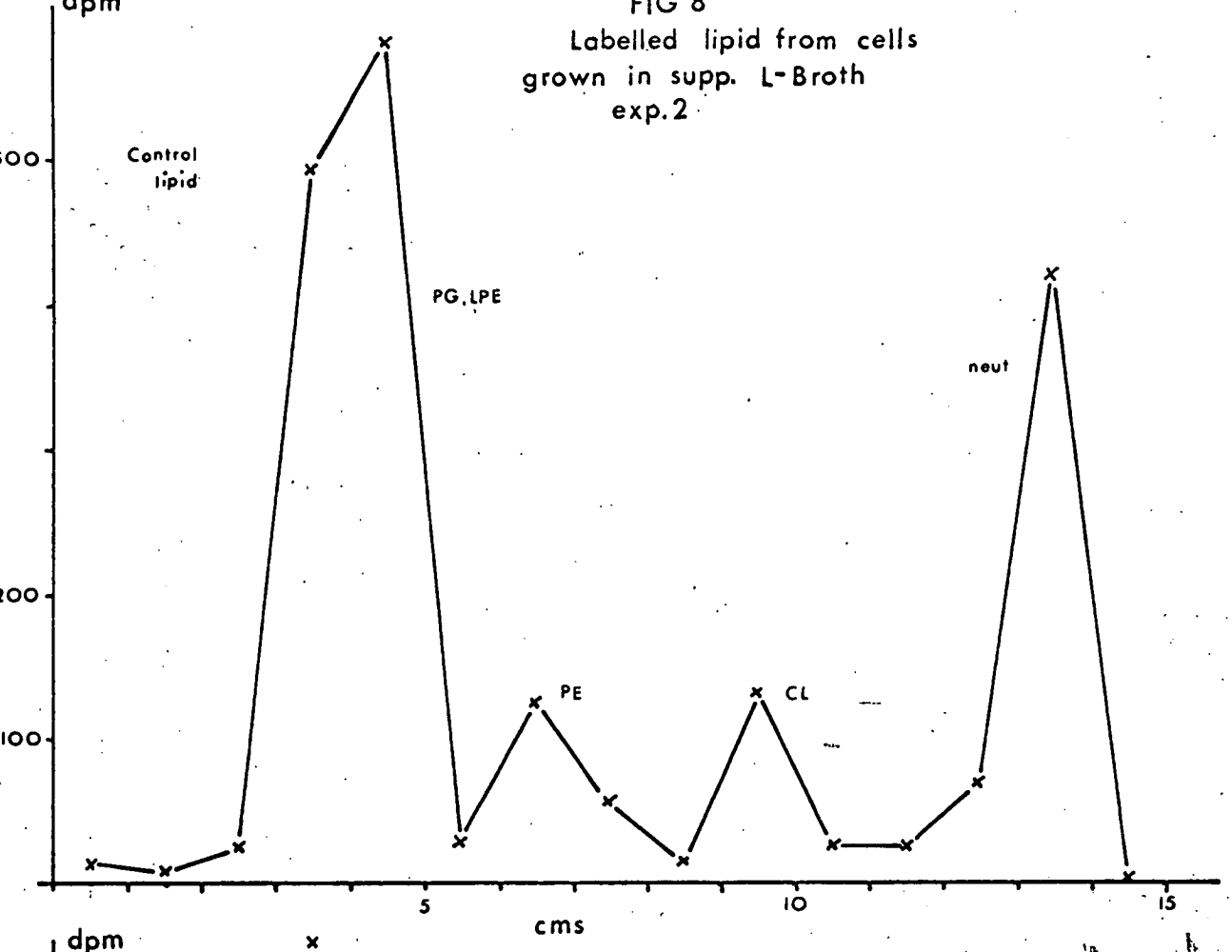
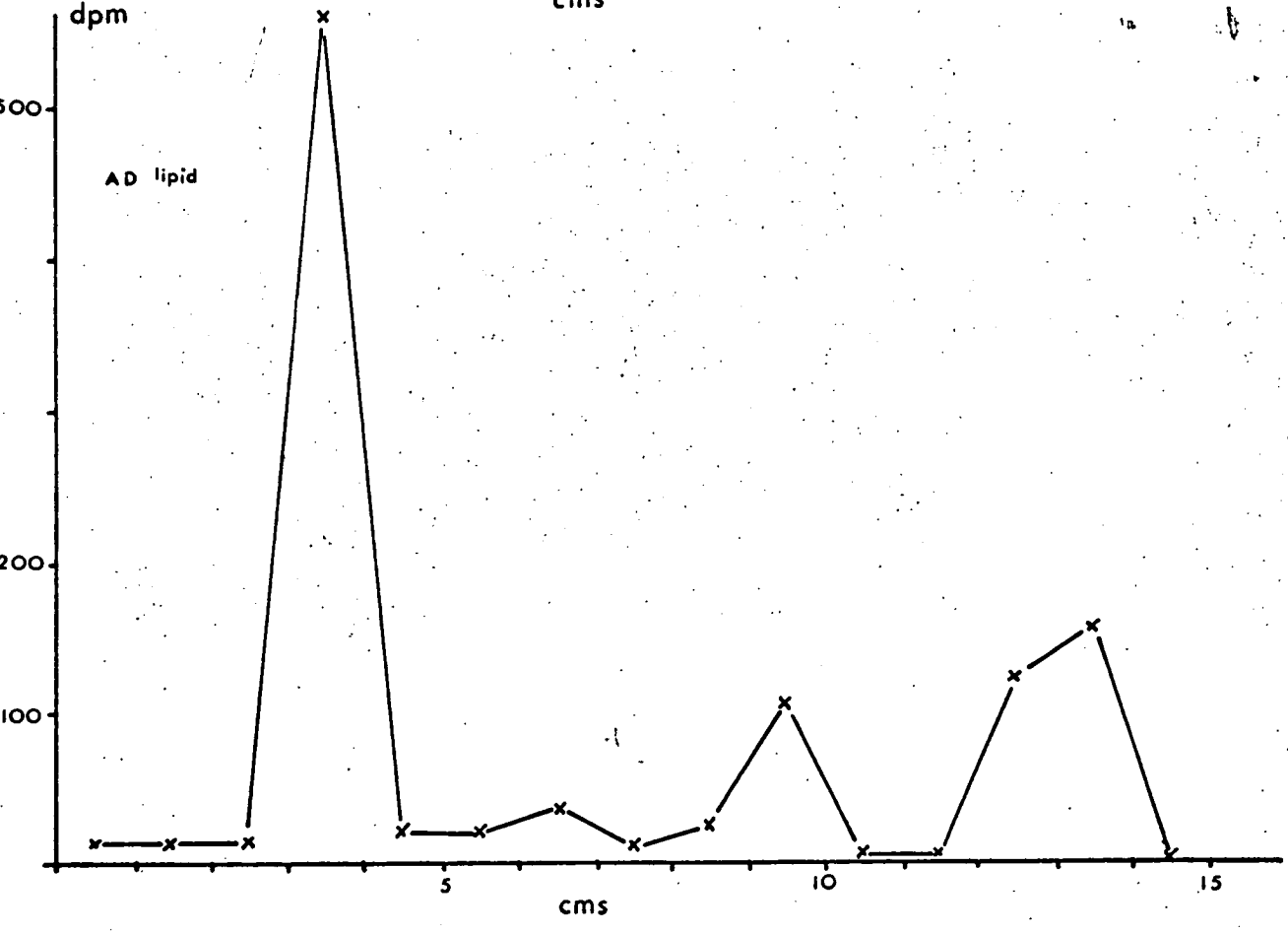

Incorporation of ^{14}C leucine into 5% TCA precipitates
dpm
10^4
10^3
10^2
Control, unsupp. L-Broth (0·3→1·2)
Control, supp. L-Broth (0·17→0·63)
AD treated unsupp. L-Broth (0·25→0·22)
AD treated supp. L-Broth (0·15→0·13)
10
20
30
40
min.

FIG 8

Labelled lipid from cells grown in supp. L-Broth exp. 2





components corresponding closely to those of control cell lipid, irrespective of the O.D. at which experiments were conducted. No change was evident in the lipids labelled at high (1.2) and low (0.4) O.D.s in control cells, but difficulty was encountered in comparing different sets of experiments as the gels did not run very reproducibly (Figs. 7, 8), noted previously in the chemical studies (p.93).

Residual protein synthesis during A.D. treatment

Figure 9 shows the incorporation of ^{14}C leucine with time into 5% TCA precipitates of cells grown with and without A.D. in the two media; commencing and final O.D.s are given in brackets. In both controls, there is an exponential increase in the radioactivity counted, whereas in both A.D. treated cultures there is virtually no increase on the zero-time incorporation.

Effect of lipid extraction on cell morphology

Cells fixed directly after the washing procedures for lipid extraction had normal appearances apart from some vesicularisation in the mesosomes (Plate 80). Extra membranes were not significantly affected (Plate 81). After lipid extraction, all cells had lost their membrane structures (plasma membrane, mesosomes, etc.), and the only indication of mesosomes previously present was small amorphous areas outside the cytoplasm (Plate 82) which however still appeared to retain some kind of boundary layer.

Discussion

The lipids identified in B. cereus 569 are unremarkable, and have been reported in other strains of B. cereus (Ikawa, 1967). The origin of ∝GP after deacylation in both series (Tables 2, 3) is not clear as phosphatidic acid was not identified in the lipid extracts; a possibility is that some hydrolysis of GPGPG occurred during the highly alkaline conditions of methanolic deacylation.

The results of the experiments using tritiated glycerol show that the label is incorporated both into lipid and cell residue during A.D. treatment (Fig. 5); during this treatment, however, there is virtually no growth as measured by O.D. nor protein synthesis as measured by ^{14}C leucine uptake (Fig. 9). The nature of the cell residue which incorporates tritiated glycerol is presumed to be wall teichoic acid, but there is little doubt that activity in the lipid fraction is contained in complexed components that run very closely with control cell components and correspond to normal phospholipids. As the amounts of lipids involved in these experiments was too small for detection by spraying, and the quantities could not be scaled up with the available A.D., it is not possible to usefully comment on changes in the elution profiles in some gels, for example Figure 7, except that one of the slower moving components appears to be consistently absent or reduced (Figs. 6, 7, 8).

No change in lipid composition of control cells was observed on entry into lag phase either by chemical identification or radio-active lipid profiles. Lang (1970) found no qualitative change in the phospho lipids of B. cereus ATCC 4342 during growth and sporulation, although quantitative changes occurred.

Finally, the site of lipids extracted appears to be in part at least the plasma-membrane and mesosomes, as demonstrated by the electron microscope. Thus a consideration of all the evidence presented here and in previous sections lends very strong support to the view that during A.D. treatment membranes continue to be produced in B. cereus 569, and teichoic acid synthesis is not inhibited. The nature of the membranes produced is uncertain, and in the absence of protein synthesis may be phospholipid "myelin forms". They do, however, have electron-microscope characteristics of natural membranes, and it is therefore possible that they may be of similar structure to normal membranes, the proteins being supplied from an endogenous pool. Attempts were made to isolate labelled membranes without much success, partly due to contamination by label in the wall that was not prevented by growth in phosphate and magnesium supplemented medium, and partly due to the resistance of the cells to lysosyme. These investigations are presented in a short form in the Appendix following. However, it would appear that phosphate and magnesium supplementation reduces considerably the wall glycerol

teichoic acid (Table 5).

No conclusive evidence has been previously presented as to whether there may be more than one kind of mesosome system amongst the bacilli. To the extent that there appear to be consistent differences in the mesosome number and structure between B. licheniformis 749c and B. cereus 569, and that the control of membrane synthesis appears also different, this possibility appears to be the case, and the recognition that mesosomes may behave differently and have different functions in different species of bacilli may contribute to current approaches on their study.

APPENDIX

The protoplasting behaviour of *B. cereus* 569 and the isolation of labelled membrane fractions

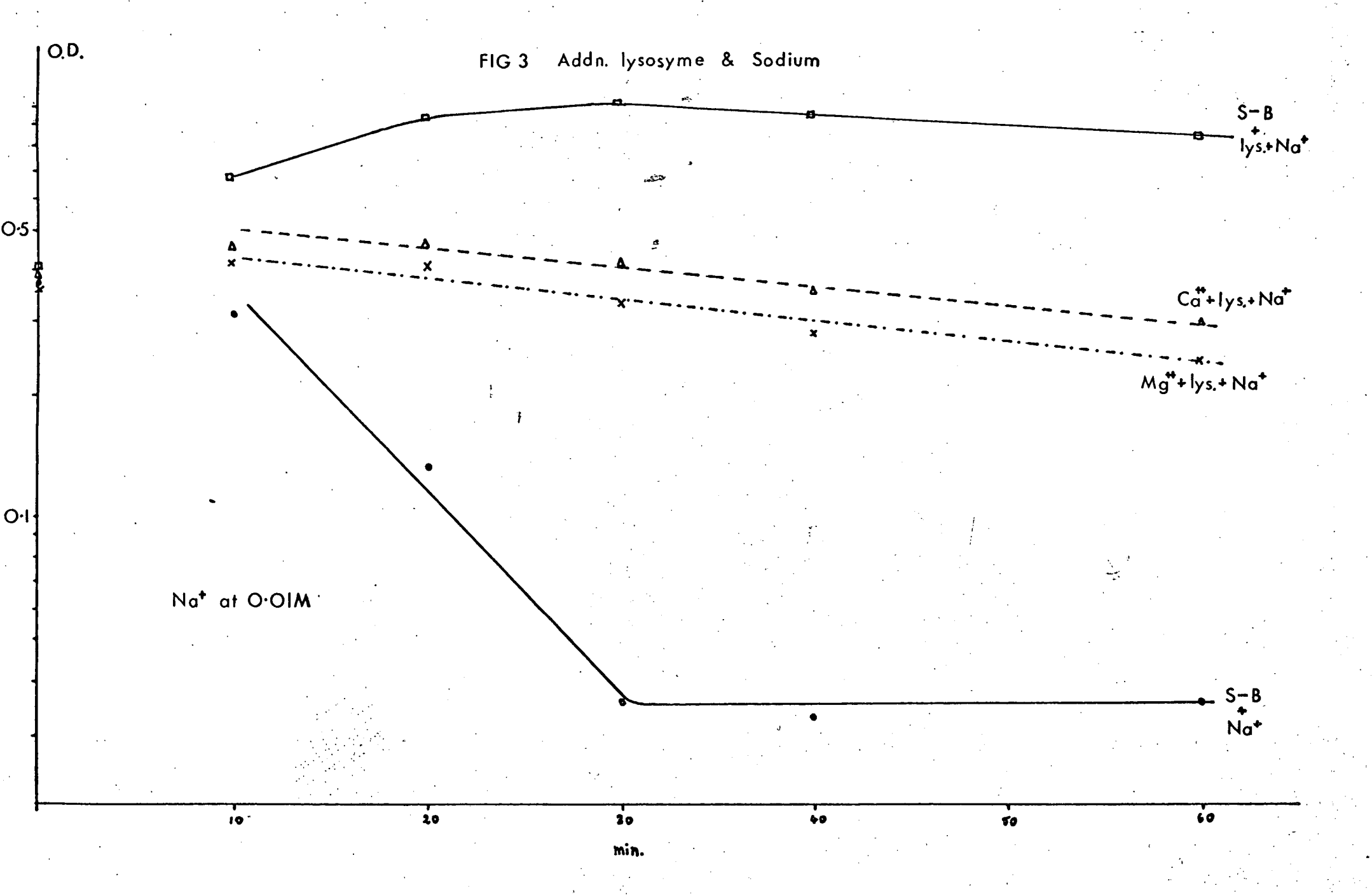


FIG 3 Addn. lysosyme & Sodium
O.D.
0·5
0·1
S–B + lys.+Na+
Ca++ + lys.+ Na+
Mg++ + lys.+ Na+
Na+ at 0·01M
S–B + Na+
10
20
30
40
50
60
min.

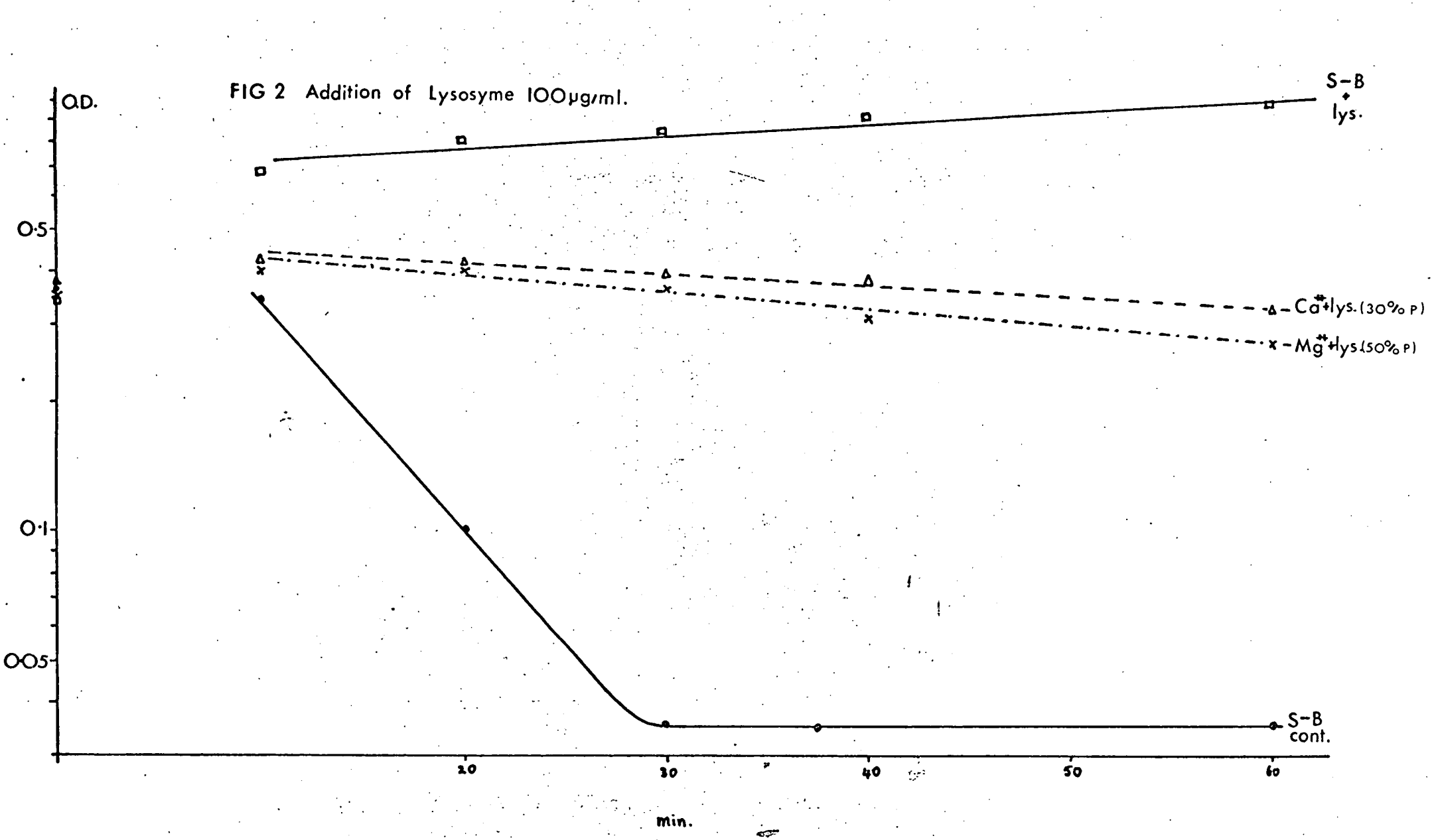


FIG 2 Addition of Lysosyme 100µg/ml.
O.D.
0·5
0·1
0·05
S-B + lys.
Δ – Ca++lys. (30% P)
x – Mg++lys (50% P)
S-B cont.
20
30
40
50
60
min.

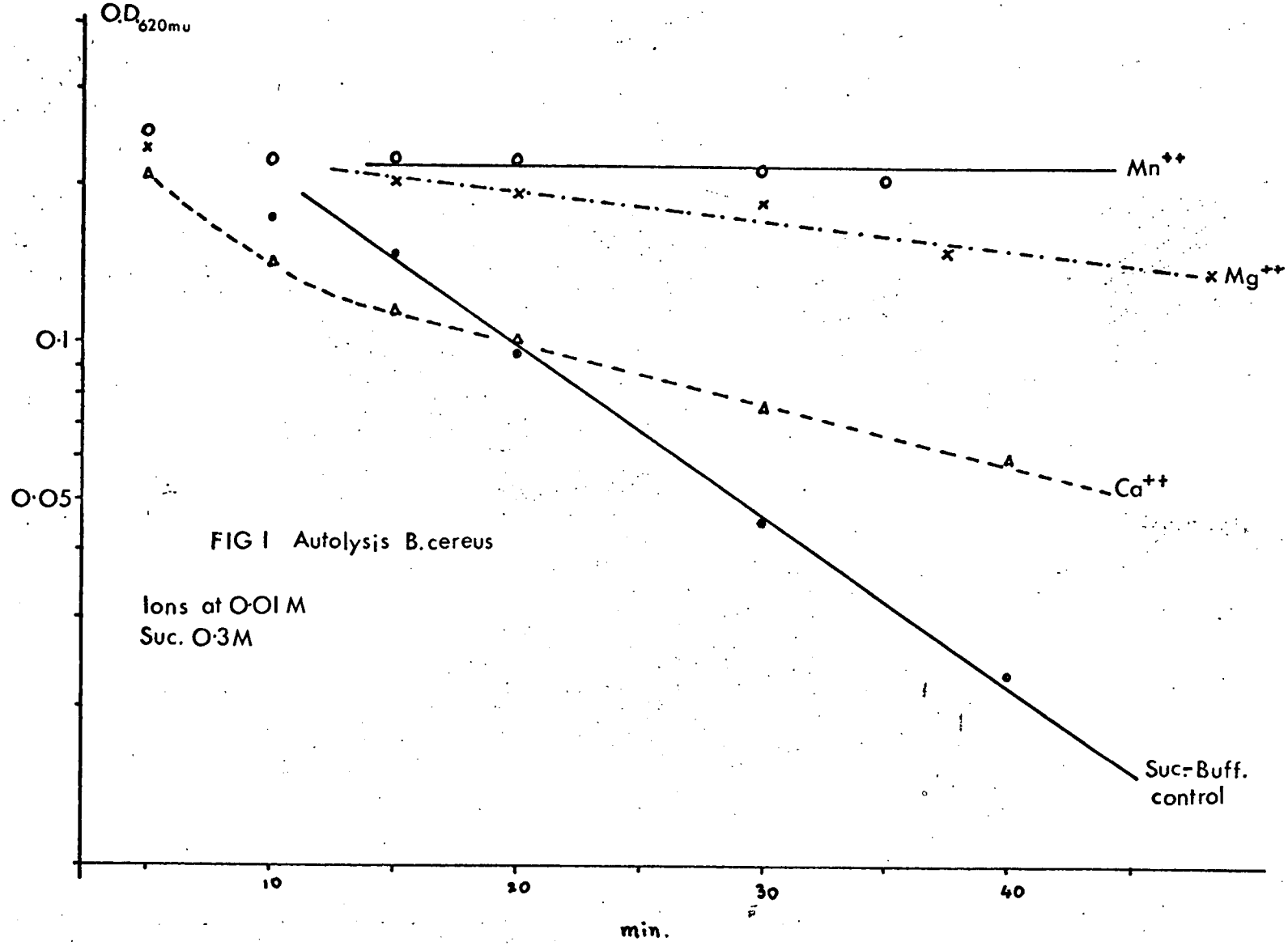


FIG 1 Autolysis B. cereus

Ions at 0·01 M
Suc. 0·3M

APPENDIX

Lysosyme in the presence of an osmotic stabiliser will produce protoplasts from most Gram-positive rods by completely removing the cell wall.

B. cereus 569 exponential cells grown in L-broth rapidly lyse when suspended at 37° in 0.01 M Tris HCl buffer at 37 pH 8 containing 0.3 M sucrose osmotic stabiliser (Fig. 1). Addition of divalent cations prevents lysis, the order of effectiveness being Mn^{++} Mg^{++} Ca^{++} (Fig. 1), but fewpprotoplasts are produced. When lysosyme at 100 ug/ml is present without cations, lysis appears to be completely prevented (Fig. 2) and phase microscopy shows apparently normal cells. Cells washed in sucrose-buffer at 4°C are still viable, and on section appear normal apart from some loss of wall material (Plate 83); cells taken from a lysosyme-sucrose buffer suspension after 20 min. are not viable but are still rod-shaped and on section have lost the wall completely, and areas of plasma membrane are missing (Plate 84). Mesosomes appear to be absent. Addition of cations with lysosyme produces protoplasts, but the maximum yield is about 50%, obtained with Mg^{++}, and further supplementation with Na^{+} makes no appreciable difference in the protoplast yield. However, the yield is raised to about 95% after 1 hour if the cells are grown in L-broth supplemented with phosphate and Mg^{++}.

Isolation of membrane fractions

Treatment of cells with A.D. in supplemented L-broth reduces the protoplast yield to about 60% (phase microscopy observations). Cells were labelled with G2T during A.D. treatment followed by treatment with lysosyme and differential centrifugation to separate protoplasts and cells from possible released membrane fragments. The following results were obtained from a typical experiment; cells were labelled at 2 uci/ml at a culture O.D. 0.47, and incubated 3 hours in lysosyme 100 ug/ml with 20% ethylene glycol stabiliser.

Fraction	Total activity (dpm)
a) Lysosome-treated culture	125,000
b) Supernate from ~~3,500 rpm~~ 2,500g x 20 min.	33,000
c) " " 30,000 g x 30 min.	24,000

Negatively-stained preparations from c) showed structures that could be interpreted as membrane fragments amongst other debris, but clean separations were never obtained even with sucrose-density-gradient centrifugations, and it was concluded that the low protoplast yield and the presence of isotope in the wall that would be released on protoplasting would effectively mask activity in labelled membrane fractions. The use of other strains and different methods may however resolve these difficulties.

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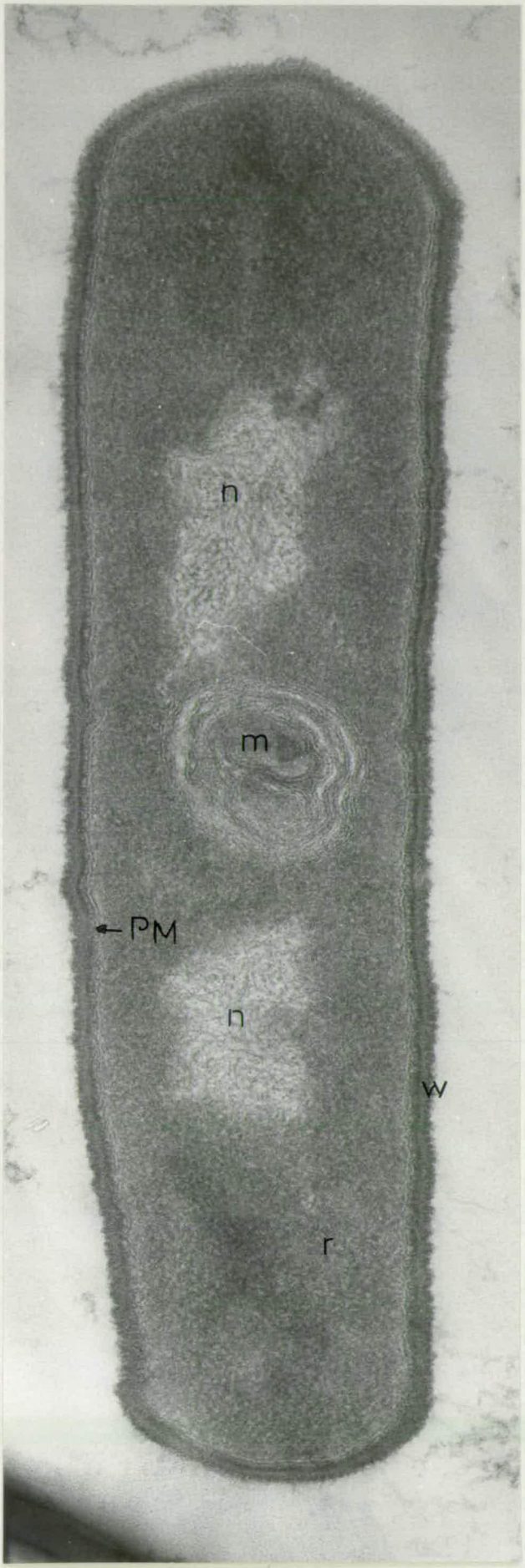


Plate 1
Vegetative cell of B. licheniformis 749c.
n = nucleus; m = mesosome; PM = plasma membrane;
w = wall; r = ribosomes.
This cell is about to commence septation as shown by the two indentations in the region of the lamellar mesosome, arrowed. x 100,000

Plate 2

Vegetative cell B. licheniformis 749c in process of division. The septum S is incomplete, and between its edges there is a large mesosome containing vesicles and lamellae. x 30,000

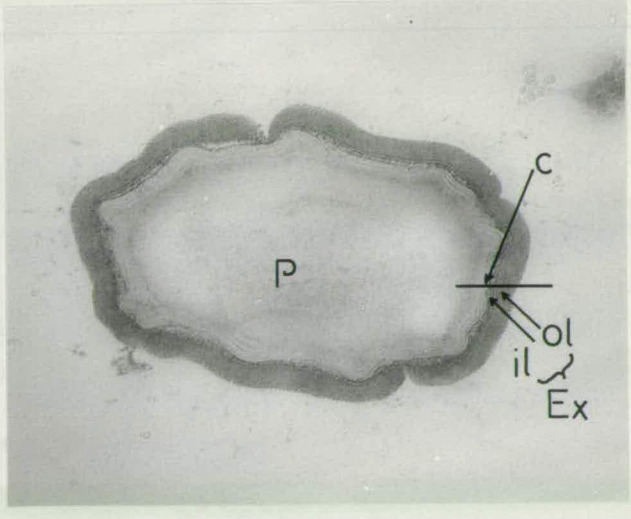


Plate 3

Ungerminated spore B. licheniformis 749c.

IL and OL = inner layer and outer layer of exosporium (Ex); c = spore coats; P = spore protoplast. x 60,000

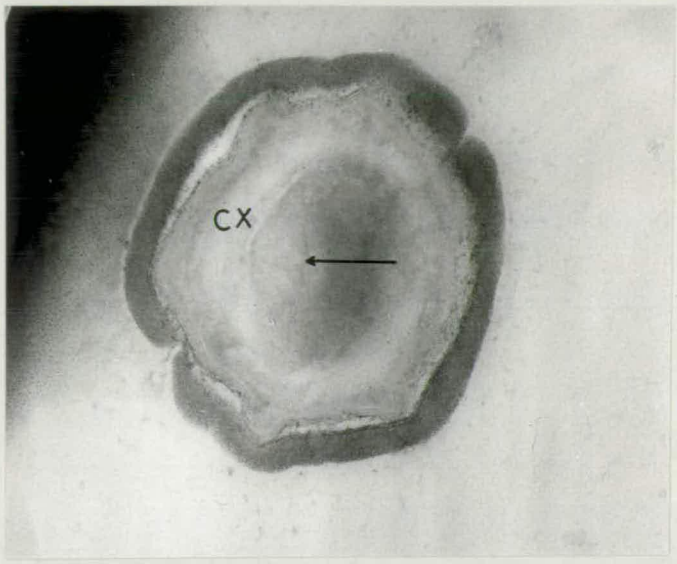


Plate 4

Ungerminated spore B. licheniformis 749c.
The protoplast has partially separated from the cortex (Cx) and a dark line appears in the protoplast, arrowed.

x 80,000

Plate 5

B. licheniformis 749c. Initial stage of germination.
The spore, protoplast ahd cortex are now clearly defined, but the protoplast still appears amorphous.
30 min. alanine-inosine. x 90,000

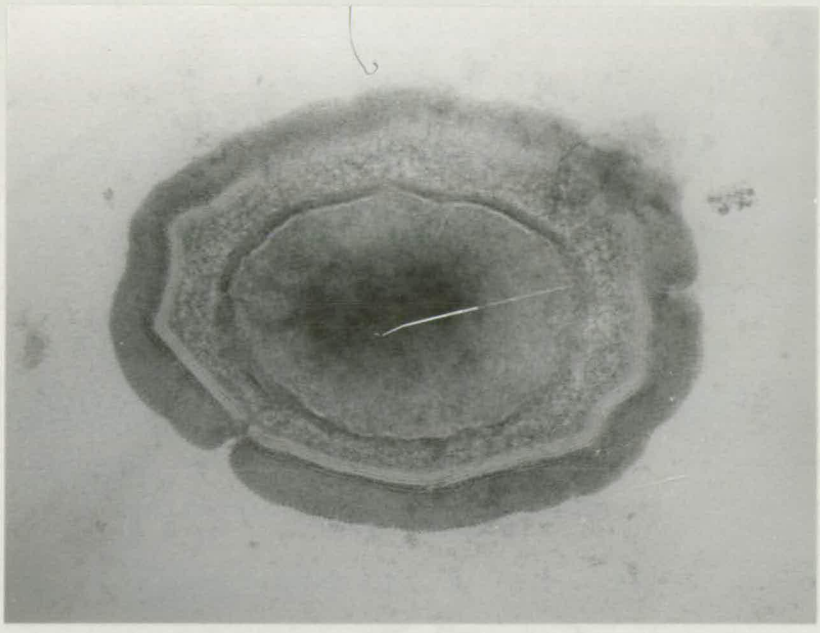


Plate 6
Germination of B. licheniformis 749c.
The cortex is now reticulated and a dark layer surrounds the protoplast. 30 min. alanine-inosine. x 90,000

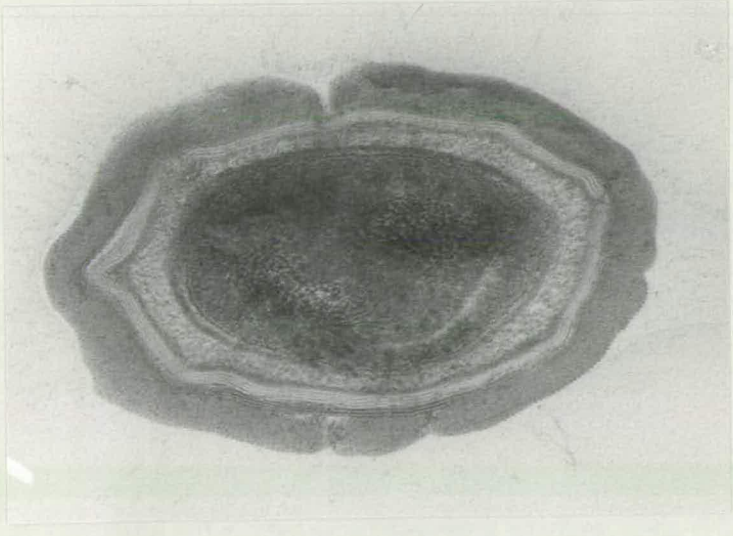


Plate 7
Germination of B. licheniformis 749c.
The protoplast is becoming hydrated, and the plasma membrane is clearly defined. Structure is visible in the protoplast interior. 90 min. alanine-inosine.
x 84,000

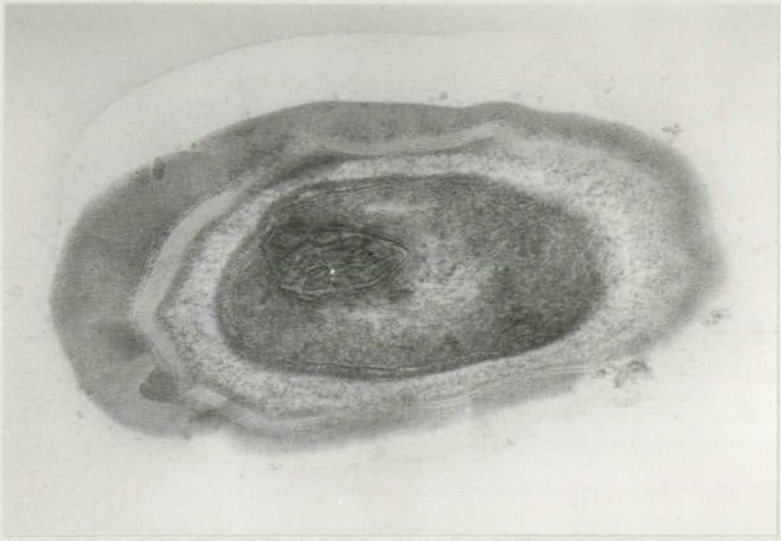


Plate 8
Germination of B. licheniformis 749c.
Membranous inclusion in early germination.
90 min. alanine-inosine. x 84,000

Plate 9
Germination of B. licheniformis 749c.
Full hydration of core. The cortex is nearly empty, and the protoplast has DNA well defined.
90 min. alanine-inosine. x 80,000

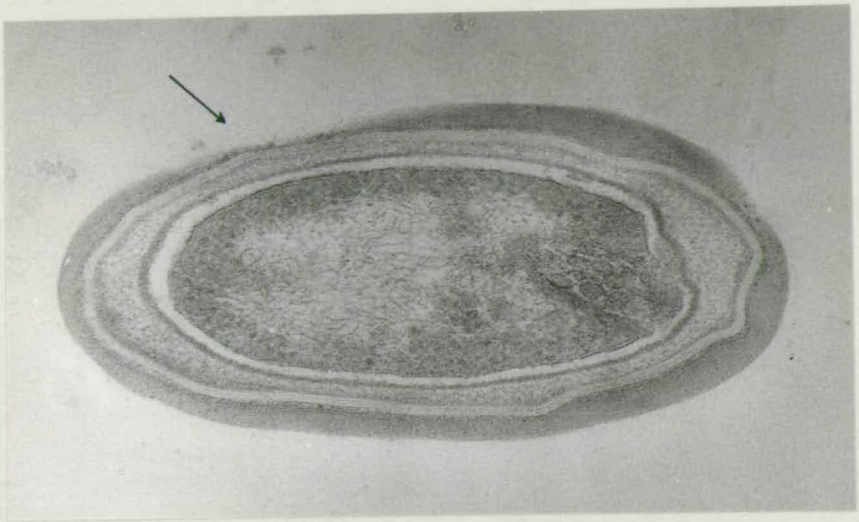


Plate 10
Germination of B. licheniformis 749c.
Membranous inclusions in late germination. The exosporium is beginning to dissolve, arrowed.
8 hours alanine-inosine. x 80,000

Plate 11
Germination of B. licheniformis 749c.
Membranous inclusions in late germination.
8 hours alanine-inosine. x 80,000

Plate 12

Doubtful membrane structures in late germination. Such appearances could be sections of finger-like processes from the protoplast.

8 hours alanine-inosine. x 80,000

Plate 13

Germination of *B. licheniformis* 749c.

Lamellar membrane structures in late germination.

8 hours alanine-inosine. x 80,000

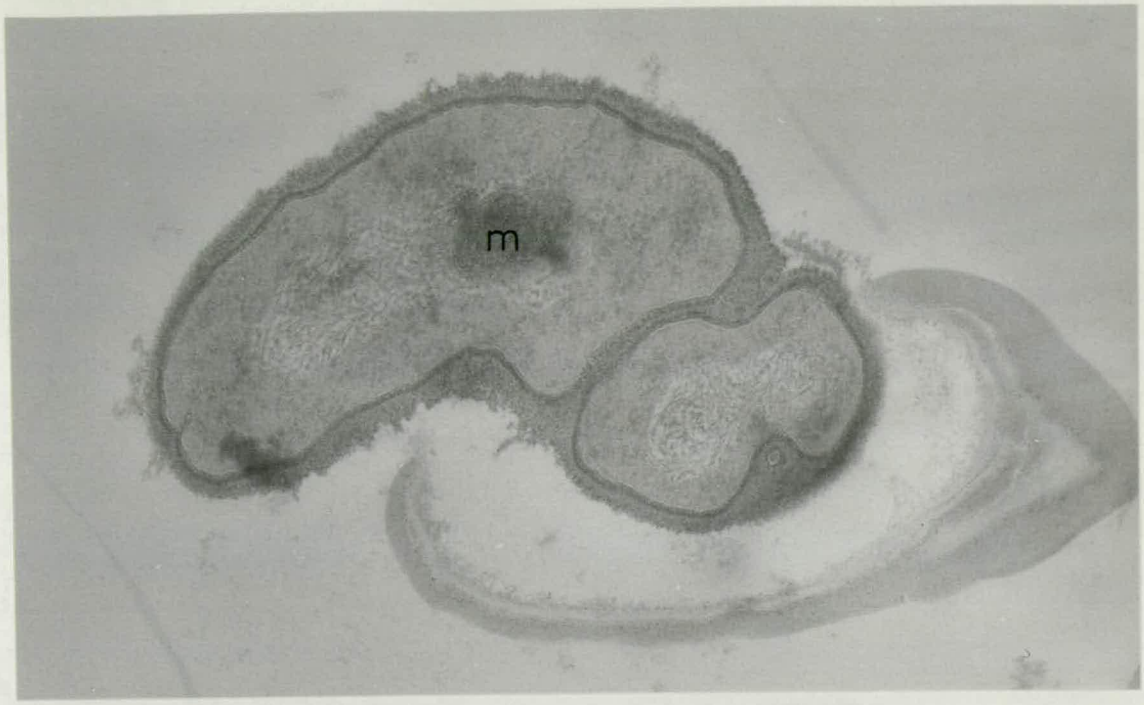


Plate 14

Outgrowing cell after 8 hours germination in alanine-inosine. Walls are thickened and distorted, and the ribosome content of the protoplasm poor (compare with Plates 1 and 2). A membranous structure similar to a mesosome is present (m) and a cell division has occurred as shown by the complete septum. x 80,000

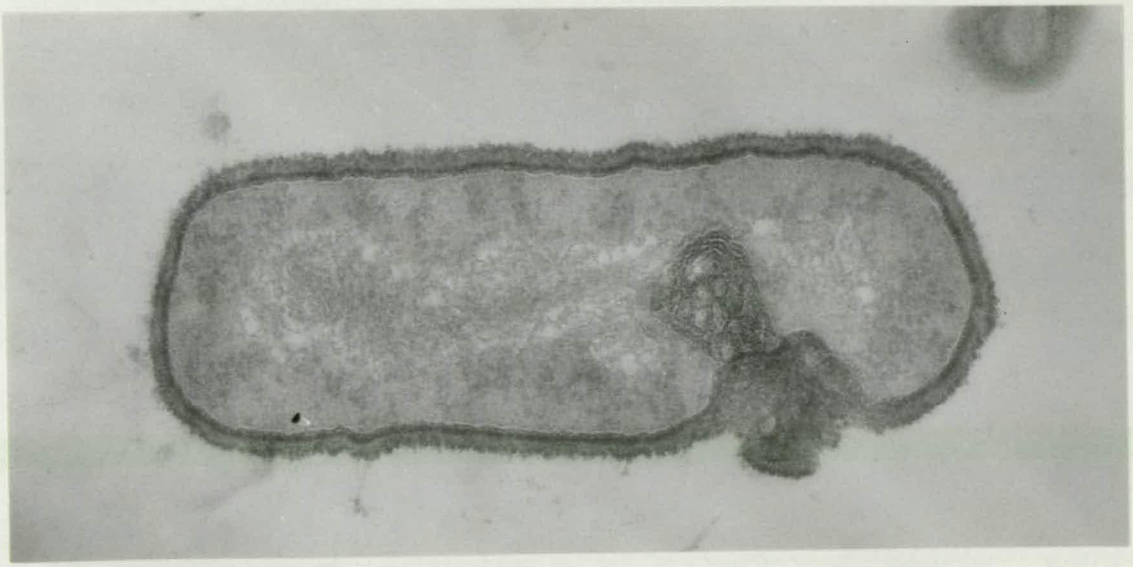


Plate 15

Vegetative cell B. licheniformis 749c.
20 min. after transfer to outgrowth medium. x 60,000

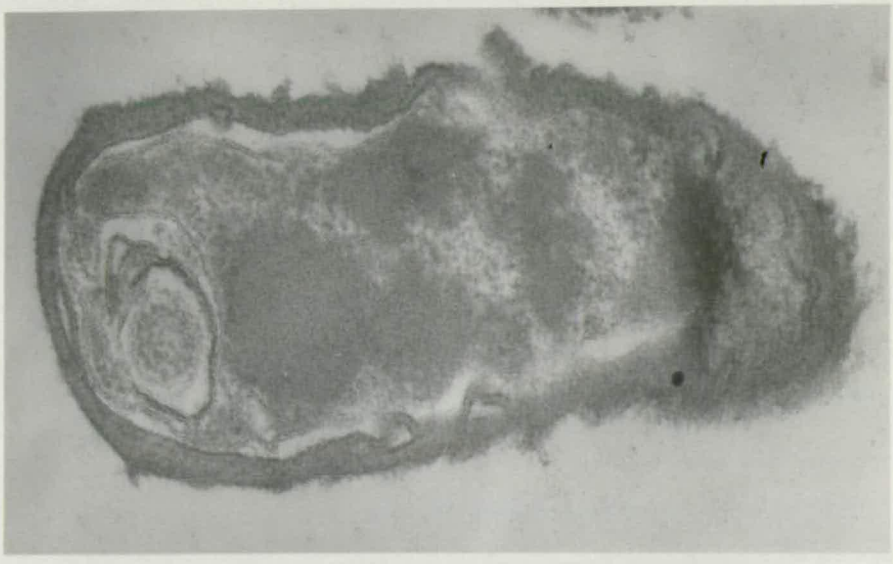


Plate 16
B. licheniformis 749c. Disintegrating cell, 30 min. after transfer to outgrowth medium. x 80,000

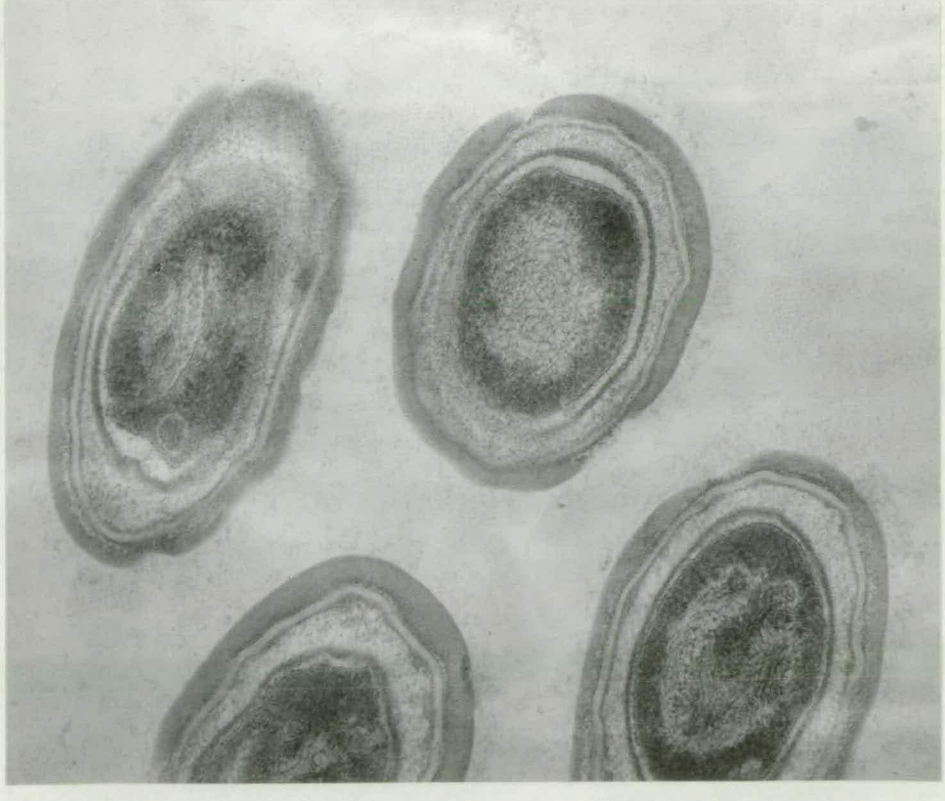


Plate 17
B. licheniformis 749c. Spore field, 30 min. after transfer to outgrowth medium. x 60,000

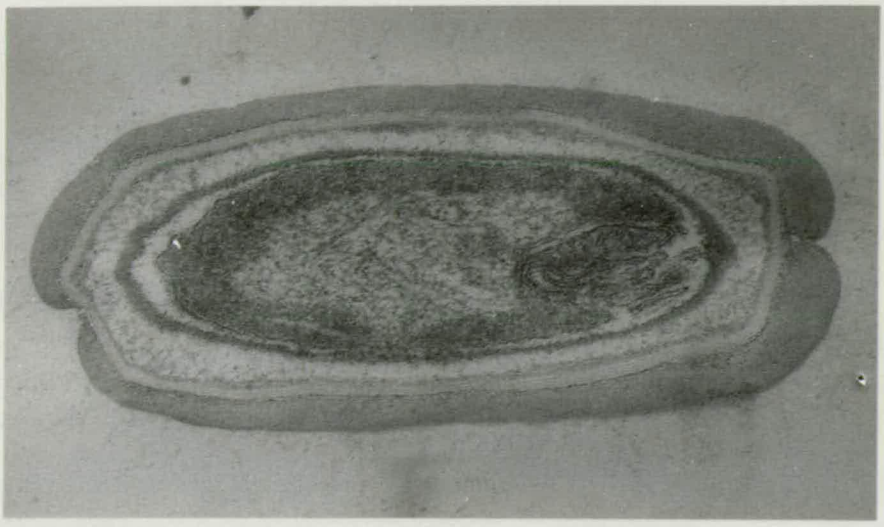


Plate 18

B. licheniformis 749c. 60 min. in outgrowth medium.
Lamellar membrane collection. x 80,000

Plate 19

B. licheniformis 749c. 60 min. germination in L-broth.
x 84,000

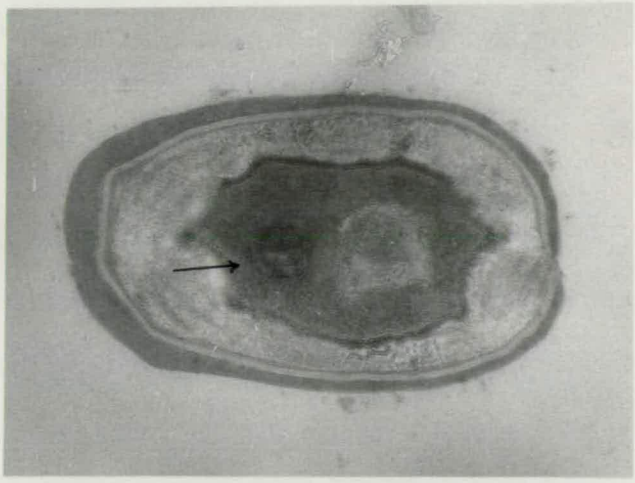


Plate 20

60 min. germination in L-broth. An ill-defined inclusion is present, arrowed. x 60,000

Plate 21

Lamellar mesosome before vegetative outgrowth has commenced. x 60,000

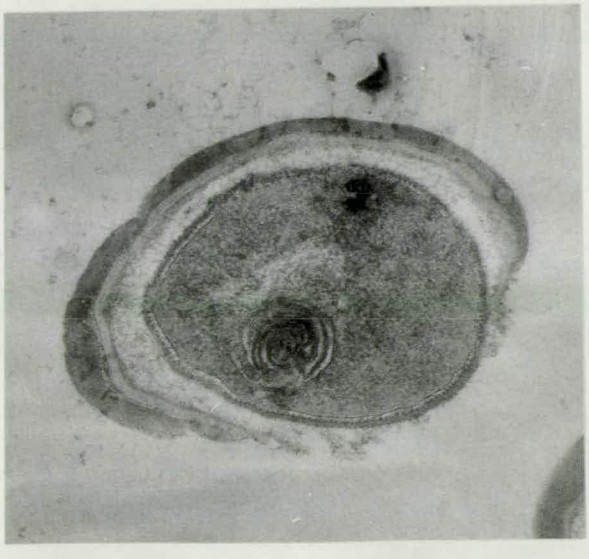


Plate 22

Lamellar mesosome in early vegetative outgrowth. x 60,000

Plate 23
60 min. germination in L-broth. Emergence of vegetative cell with mesosome. x 84,000

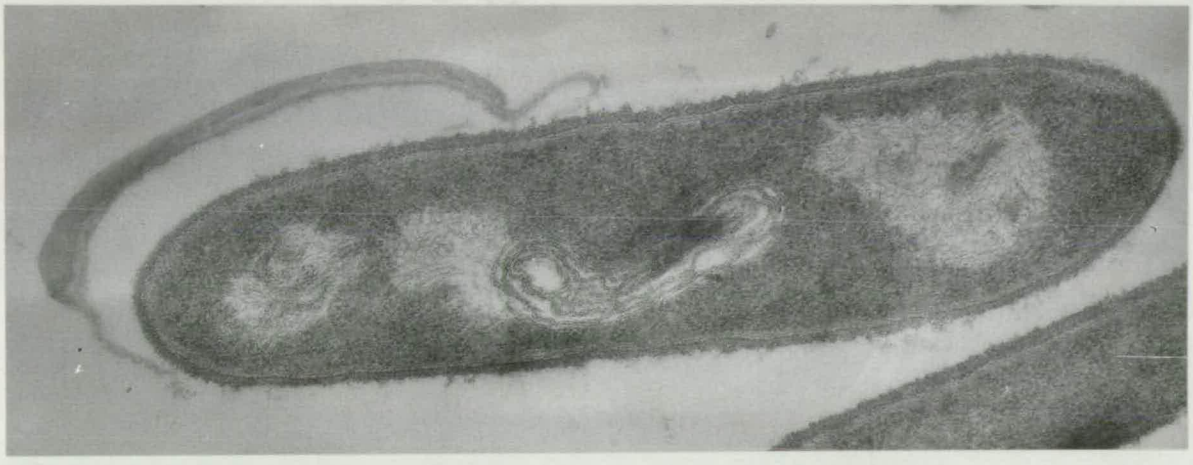


Plate 24
Spore remnant attached to vegetatively outgrown cell. 2 hrs. outgrowth in L-broth. x 60,000

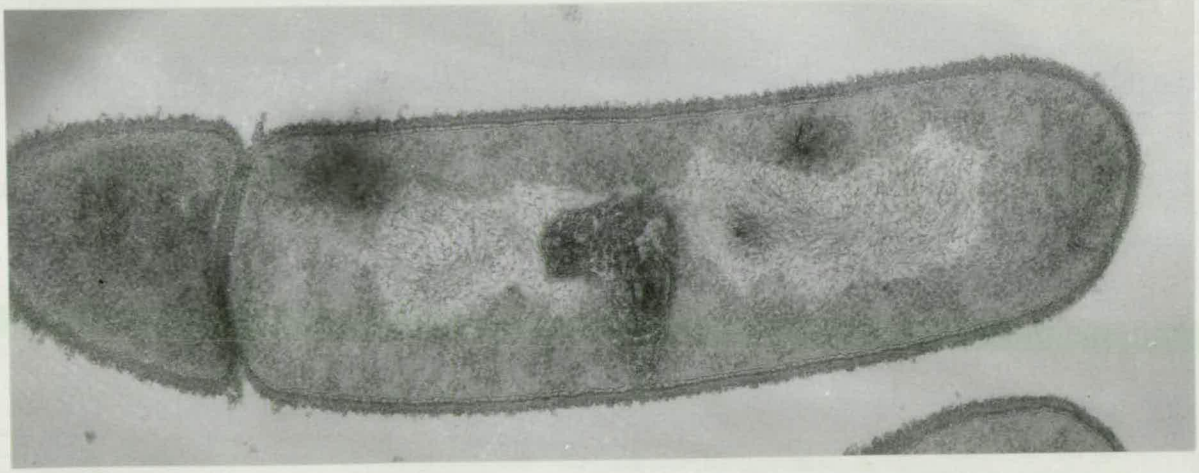


Plate 25
Free cell. 2½ hrs. outgrowth in L-broth. Part of a daughter cell is still attached; the mesosome is not very well preserved, and consists largely of vesicles.
x 60,000

Plate 26

7 hrs. outgrowth in L-broth. Cell about to divide, with a good lamellar mesosome in the septal plane.

x 60,000

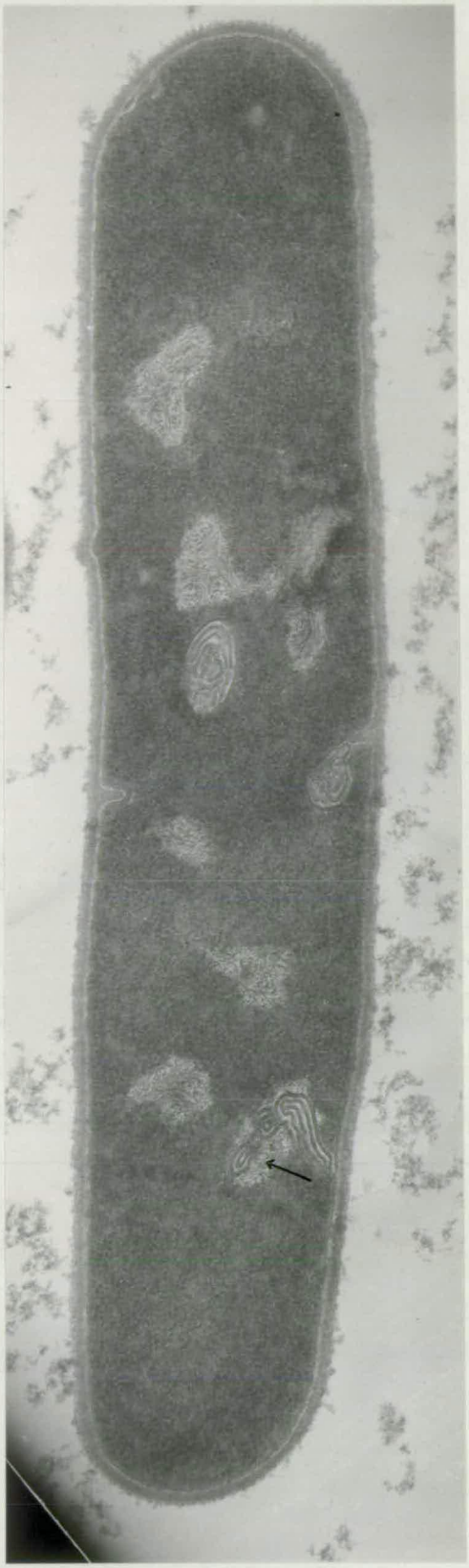


Plate 27

B. cereus 569 in L-broth, O.D. 0.35.
The DNA is scattered, and 3 mesosomes are present, 2 of them lamellar-vesicular. The lamella mesosome appears to be in association with the DNA (arrowed).

x 60,000

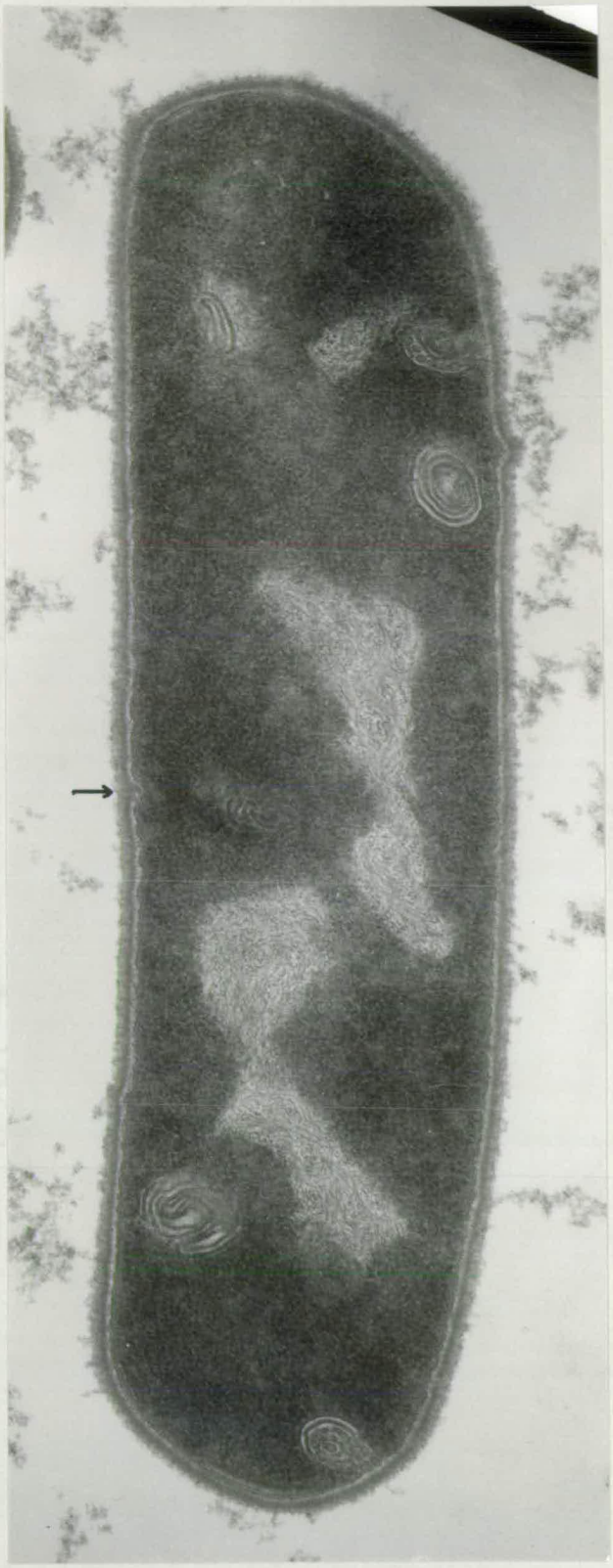


Plate 28

B. cereus 569 in L-broth at O.D. 0.35.
The DNA is more compact than in Plate 27. There are 3 lamellar mesosomes and 2 lamellar-vesicular ones; a sixth is probably present in the centre of the cell which may be commencing septation, arrowed. All such incompletely identified mesosomes were excluded in the analysis. x 60,000

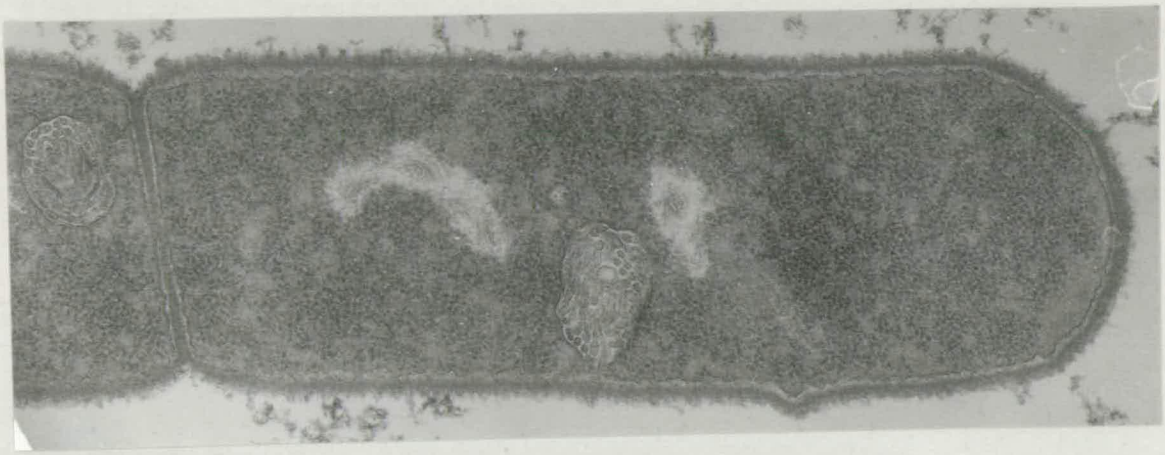


Plate 29

B. cereus 569 in L-broth, O.D. 0.35.

This cell has a dark plasma membrane and a vesicular mesosome. Triple membranes surrounding the mesosomal vesicles are difficult to discern. x 60,000

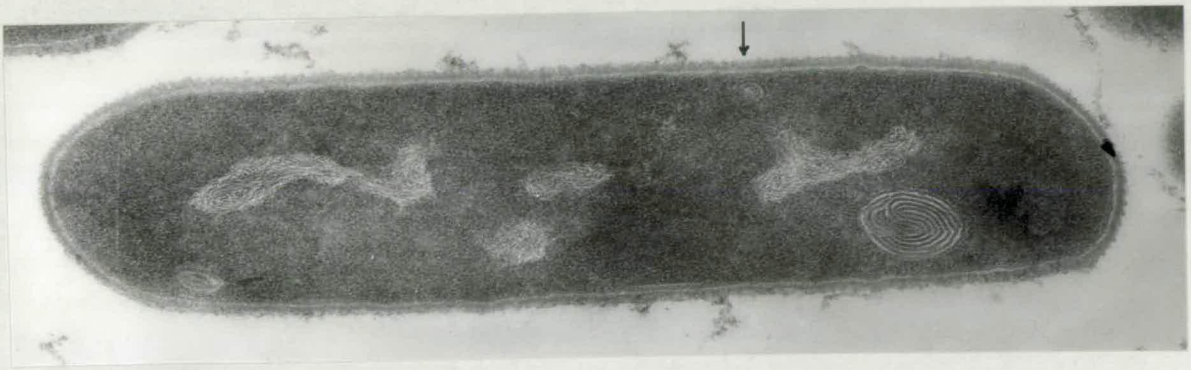
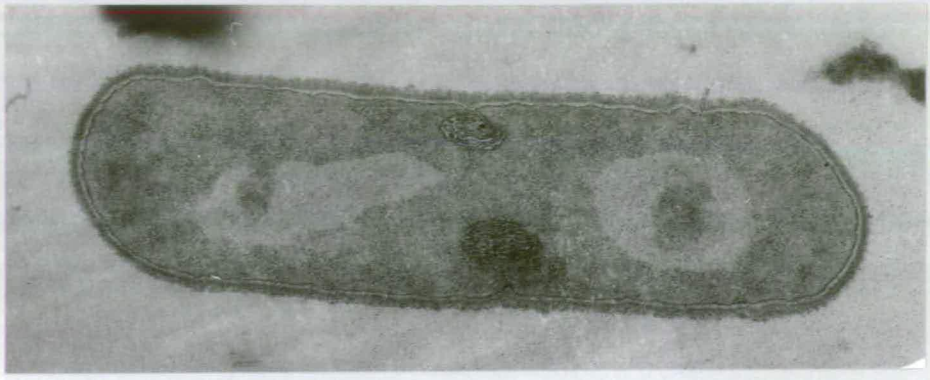


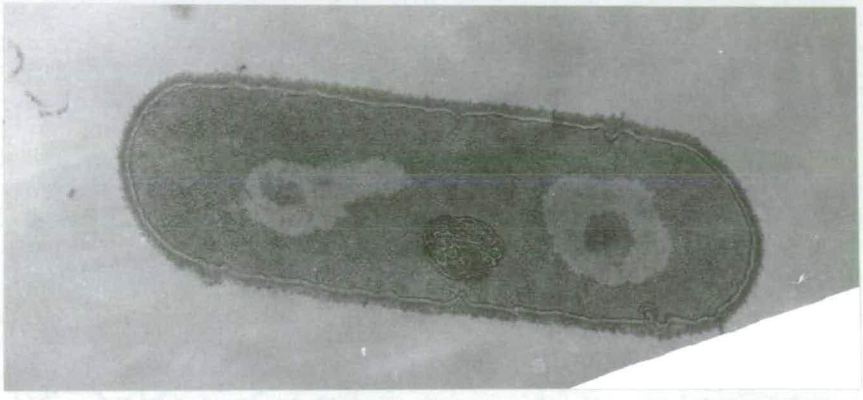
Plate 30

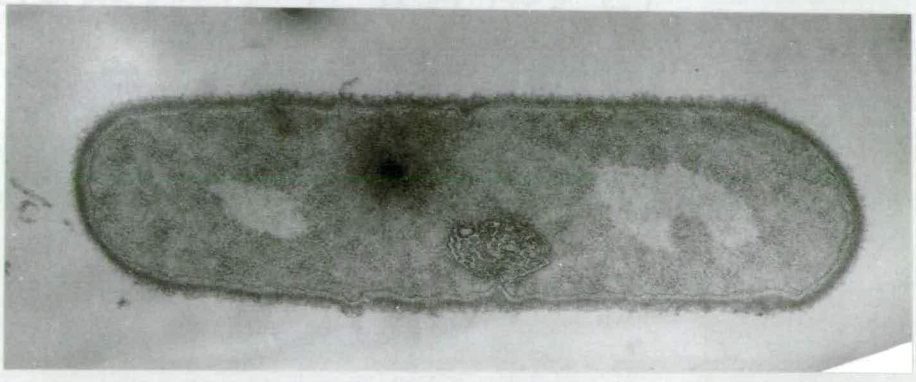
B. cereus 569 in L-broth, O.D. 0.84.

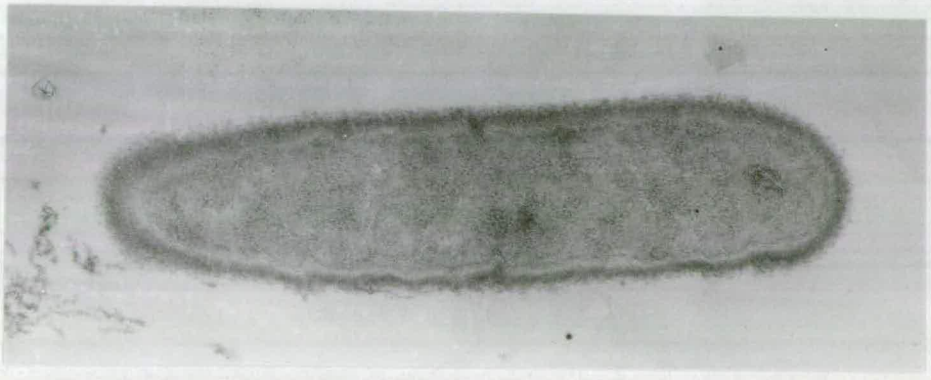
A well-developed lamellar mesosome is present together with a second at the opposite end of the cell, not so well defined. A third is probably present, arrowed.

x 40,000



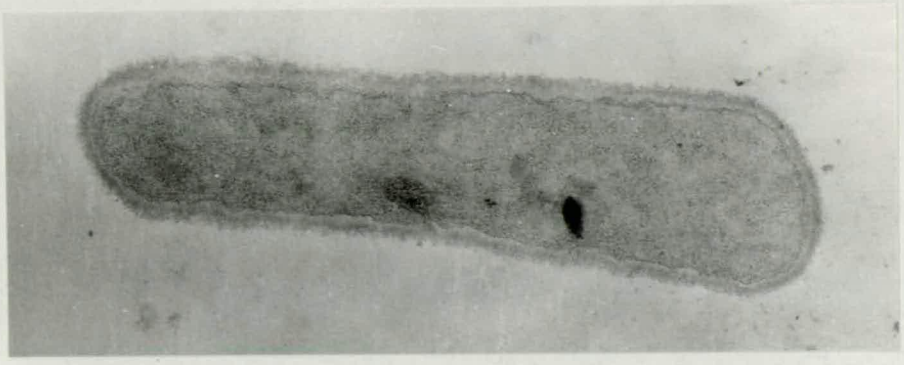


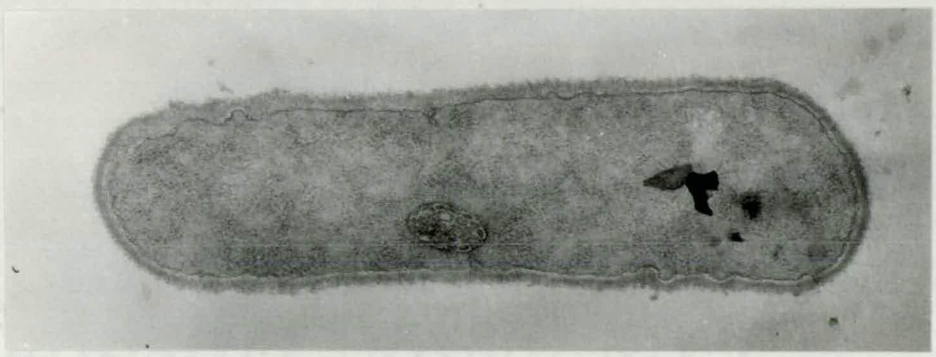


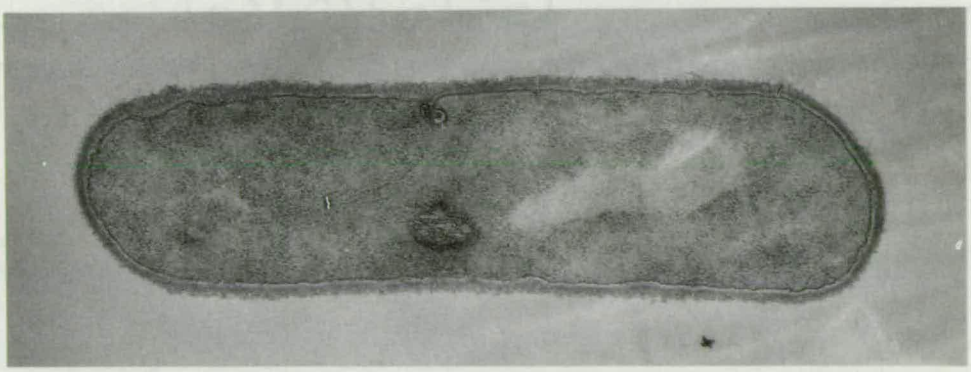


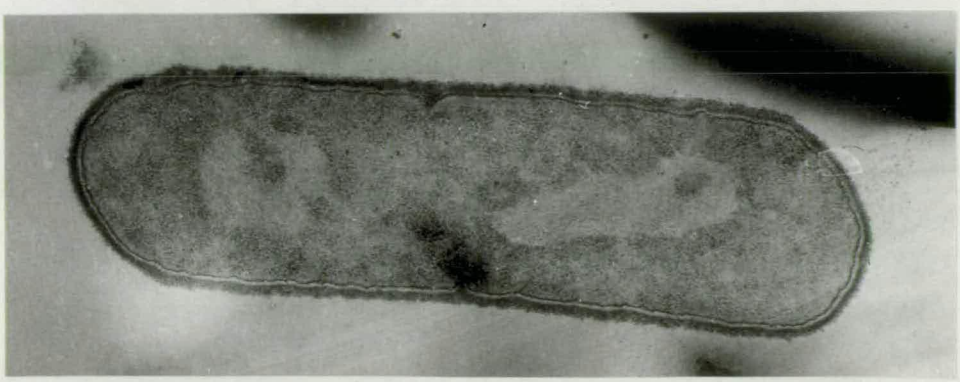
Plates 31 - 38

B. cereus 569 in L-broth, O.D. 2.0, serial section, x 40,000.
The cell has 2 vesicular mesosomes associated with the developing septum, but not in contact. The plasma membrane is dark.









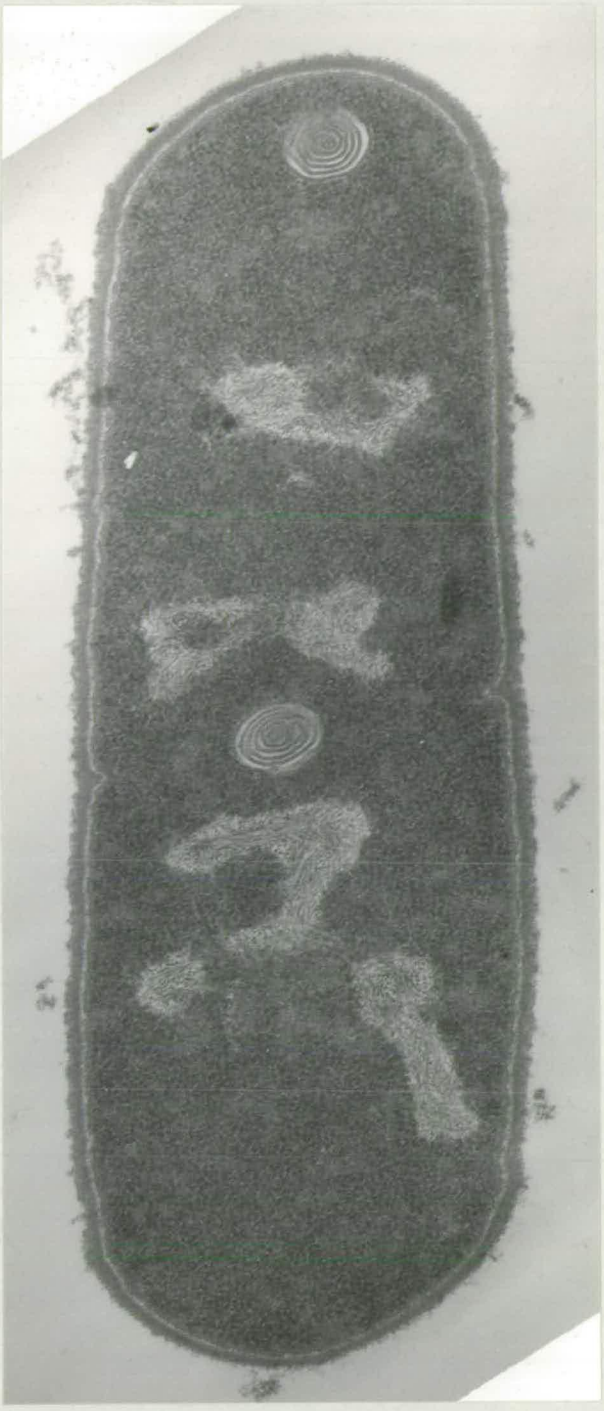


Plate 39

B. cereus in supplemented L-broth, O.D. 0.42. Mesosomes are lamellar, and there is no essential difference to Plates 27 and 28 in overall cell morphology otherwise. x 60,000

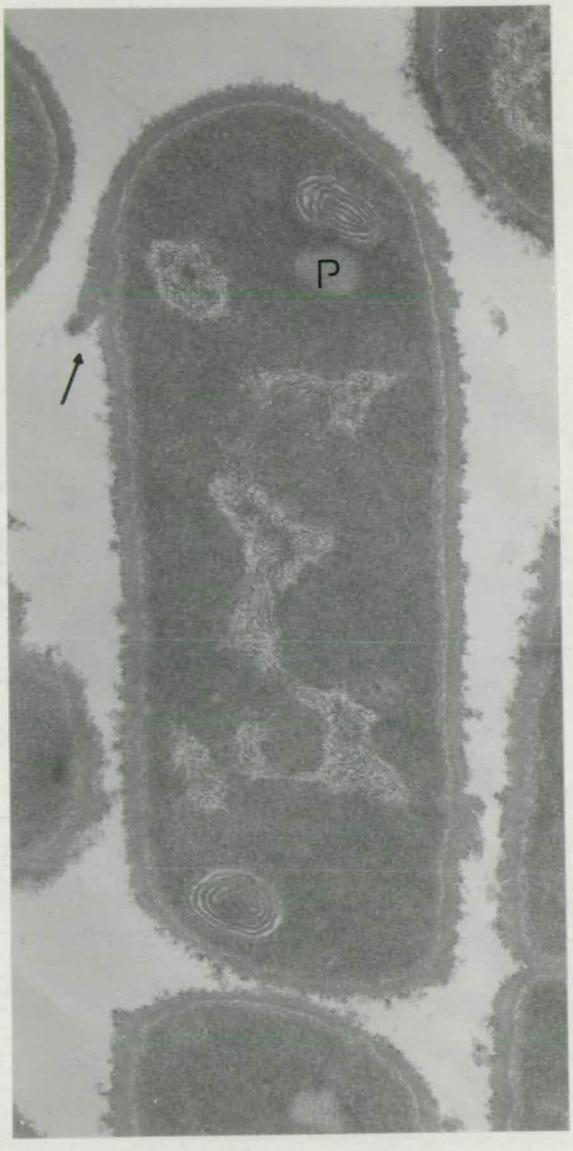


Plate 40

B. cereus 569 in Pollock's Medium, O.D. 0.31. Mesosomes are lamellar. The cell wall is thicker, and has a tendency to peel in places, arrowed. Light areas exist in the cytoplasm of many cells, one shown here, (P), which are probably polyphosphate granules. x 60,000

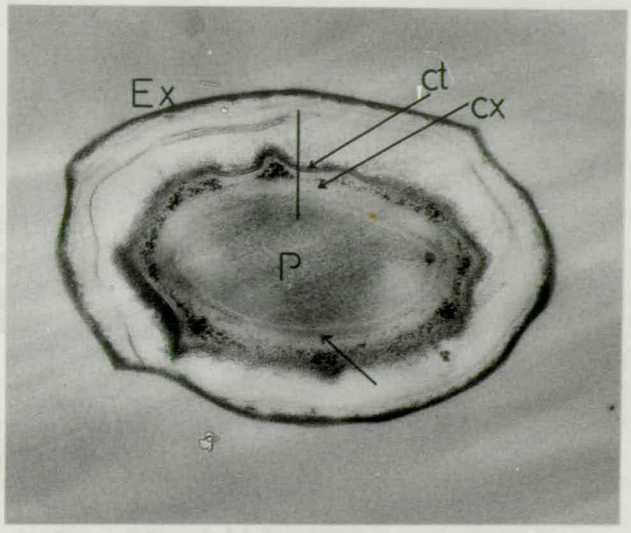


Plate 41

B. cereus 569. Dormant spore. The spore is surrounded by a loose-fitting exosporium, and a dark-staining region is arrowed in the inner part of the cortex.
Ex = exosporium; Ct = coats; Cx = cortex; P = protoplast.
x 60,000

Plate 42

Germinating spore of B. cereus 569. 15 min. alanine-inosine. The protoplast DNA ribosomes are visible and are surrounded by a plasma-membrane. The cortex is distinctly layered, and is thinner. The spore is larger, and the convolutions of the coats have disappeared. x 60,000

Plate 43

Fully germinated spore of B. cereus 569. 15 min. alanine-inosine. The swollen protoplast is cigar-shaped and smooth in outline.
x 60,000

Plate 44

Fully germinated spore. The convolutions of the spore coats have disappeared, but the surface is still apparently wrinkled, arrowed. A curved dense area is also visible in the cytoplasm. x 60,000

Plate 45

Germinated spore. An area of high contrast appears in the cytoplasm with a suggestion of a lamellar structure. x 60,000

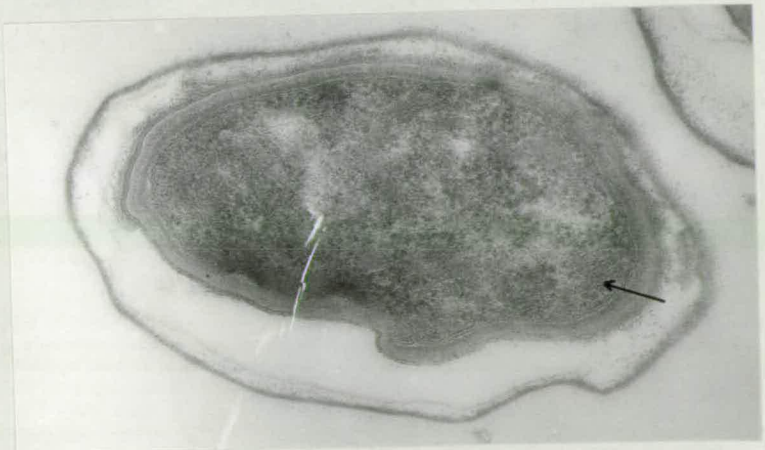


Plate 46

Germinated spore. A collection of indistinct vesicles (arrowed) lies underneath the plasma-membrane at one end of the cell, which is also wrinkled.

x 60,000

Plate 47

Membranous body after 1 hour's ~~germination~~ outgrowth in L-broth, arrowed. The DNA is also compact and centrally placed. x 64,000

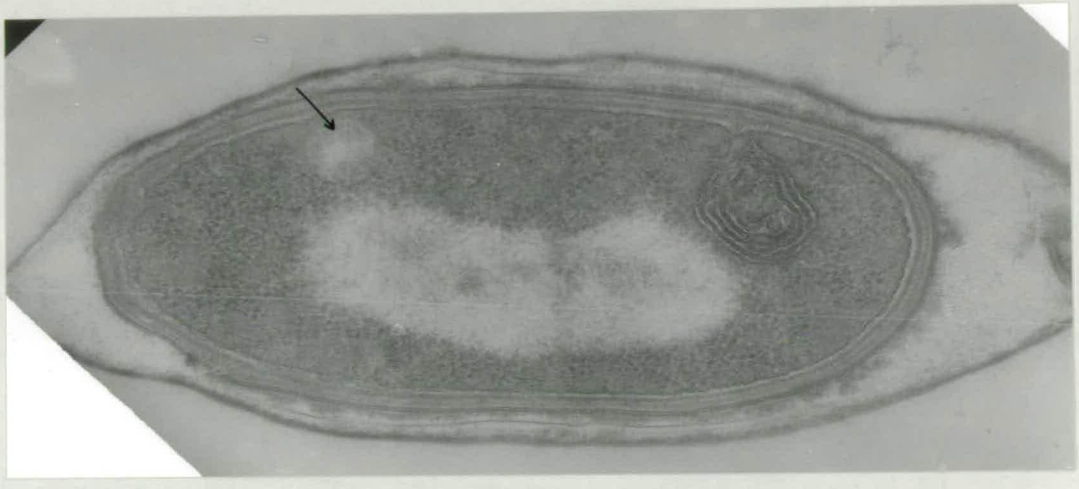


Plate 48

60 min. outgrowth in L-broth. A conspicuous mesosome is present, and is lamellar-vesicular. The light area arrowed may be another mesosome sectioned tangentially. x 64,000

Plate 49

60 min. outgrowth in L-broth. Dissolution of spore coats prior to emergence. x 60,000

Plate 50

60 min. outgrowth in L-broth. Emergence cell in the process of division. The cell has the spore case still attached, and 3 small mesosomes occur in the section.

x 40,000

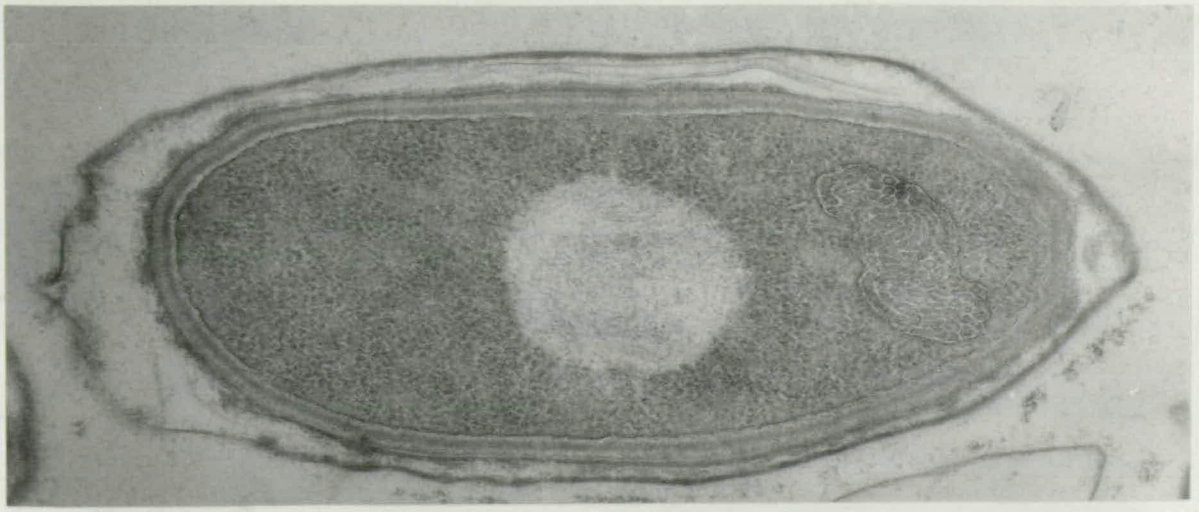


Plate 51

60 min. in L-broth + 200 ug/ml CAP. The nucleus is rounded, and a large vesicular mesosome is present. Ribosomes are plentiful. x 60,000

Plate 52

60 min. in L-broth + 2 ug/ml A.D. Membranous body, arrowed. x 60,000

Plate 53

60 min. in L-broth + 2 ug/ml A.D. The membranous body is very distinct and would be considered a mesosome. x 60,000

Plate 54

30 min. in L-broth + A.D. 2 ug/ml, followed by 30 min. in L-broth. Membranous body. x 60,000

Plate 55

30 min. in L-broth + A.D., followed by 30 min. in L-broth. Membranous body extending deeply into cytoplasm. x 60,000

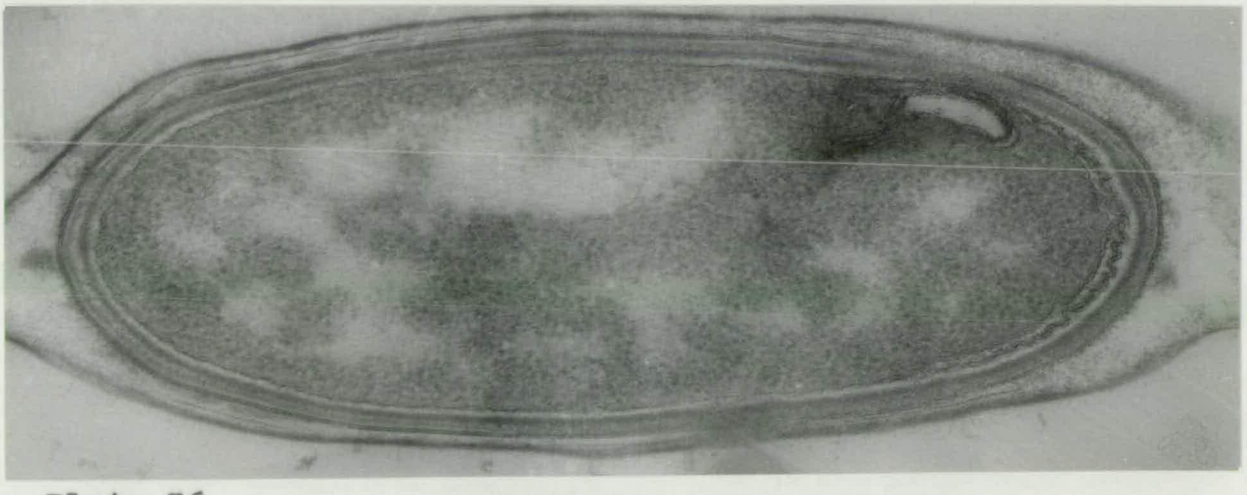


Plate 56

30 min. in L-broth followed by 30 min. in L-broth + A.D. Extra membranes appear between the plasma-membrane and the wall. The DNA is also slightly disorganised. x 60,000

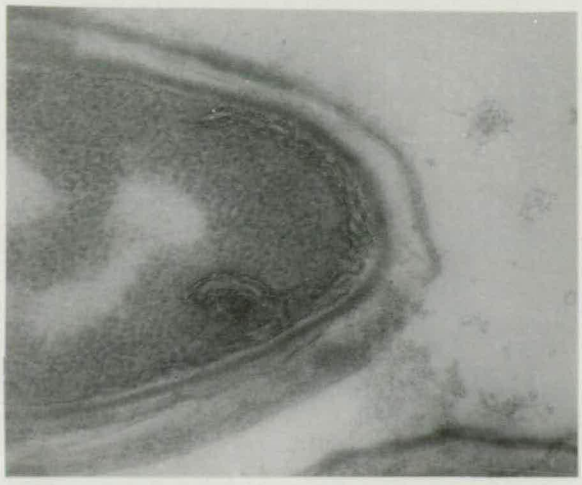


Plate 57

30 min. in L-broth followed by 30 min. in L-broth + A.D. Extra membranes appearing to emerge from a mesosome
x 84,000

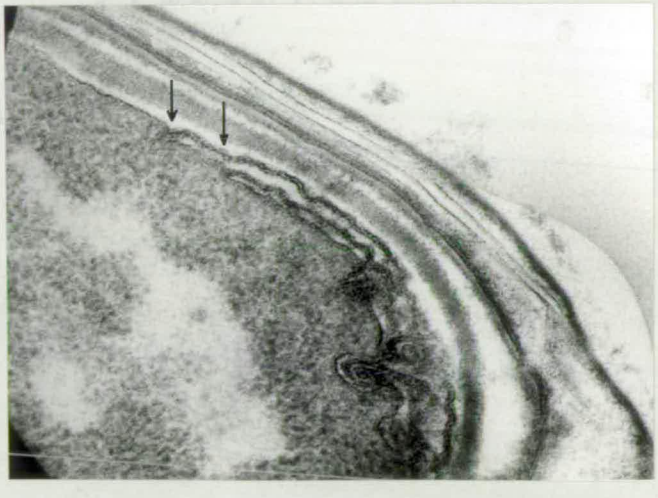


Plate 58

30 min. in L-broth followed by 30 min. in L-broth + A.D. A double layer of extra membranes is present, each layer appearing to have attachment to the plasma-membrane at arrows.

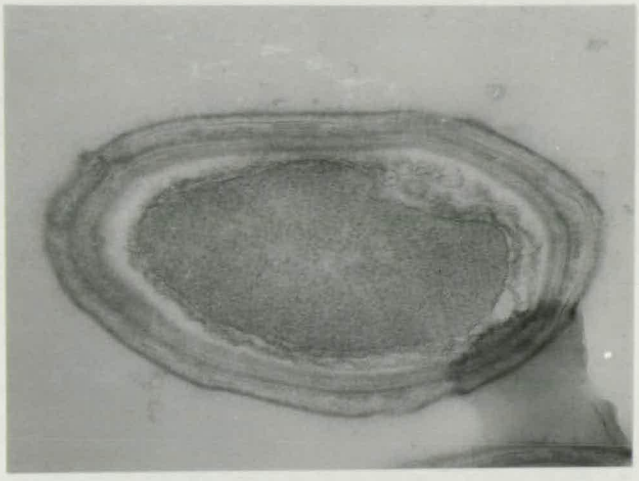


Plate 59

30 min. in L-broth followed by 30 min. in L-broth + A.D. Extra membranes completely encircling the plasma-membrane.
x 60,000

Plate 60

L-broth 30 min., acetate buffer 30 min. The mesosome is disorganised and appears to be fragmenting.
x 60,000

Plate 61

B. cereus 569. A.D. treated in buffer. 30′ x 60,000

Plate 62

B. cereus 569. A.D. treated in buffer. 30′ Degenerating mesosomes. x 60,000

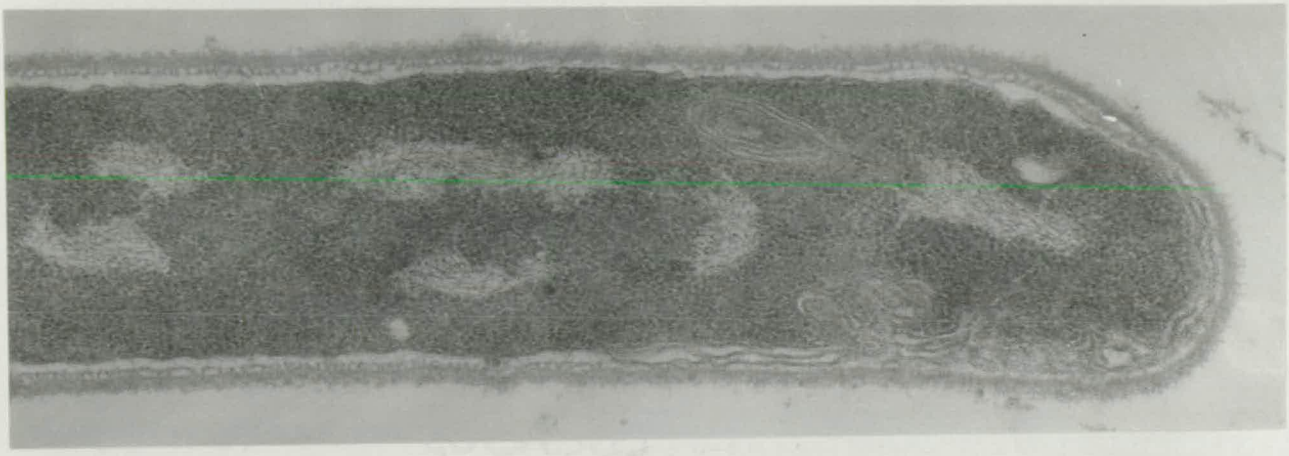


Plate 63

B. cereus 569. 30 min. treatment with A.D. Extra membranes. A second mesosome is also present. x 60,000

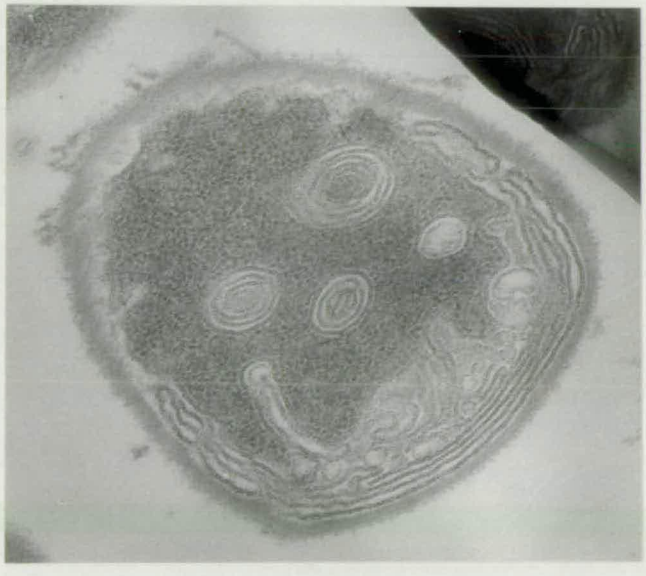
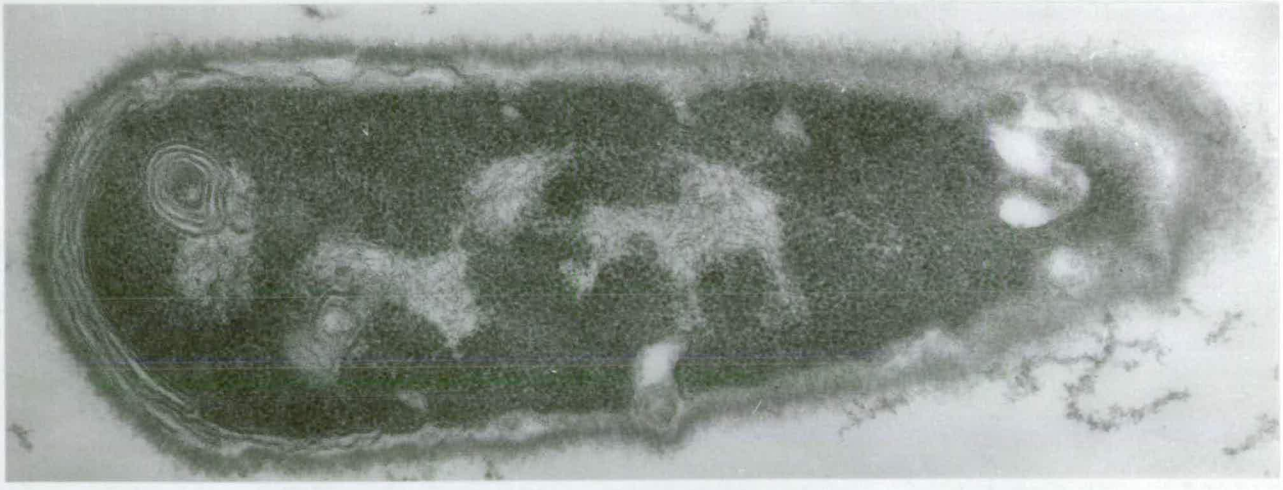


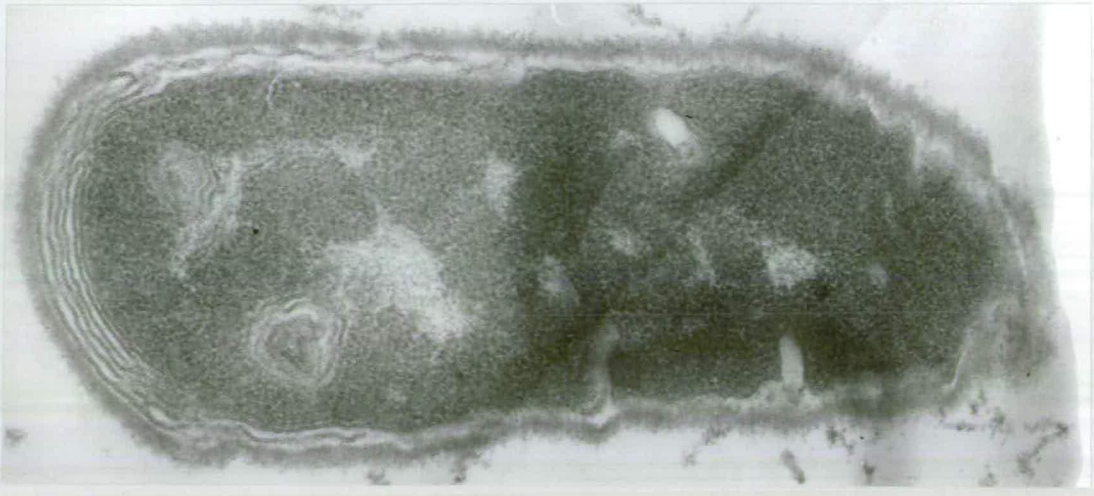
Plate 64

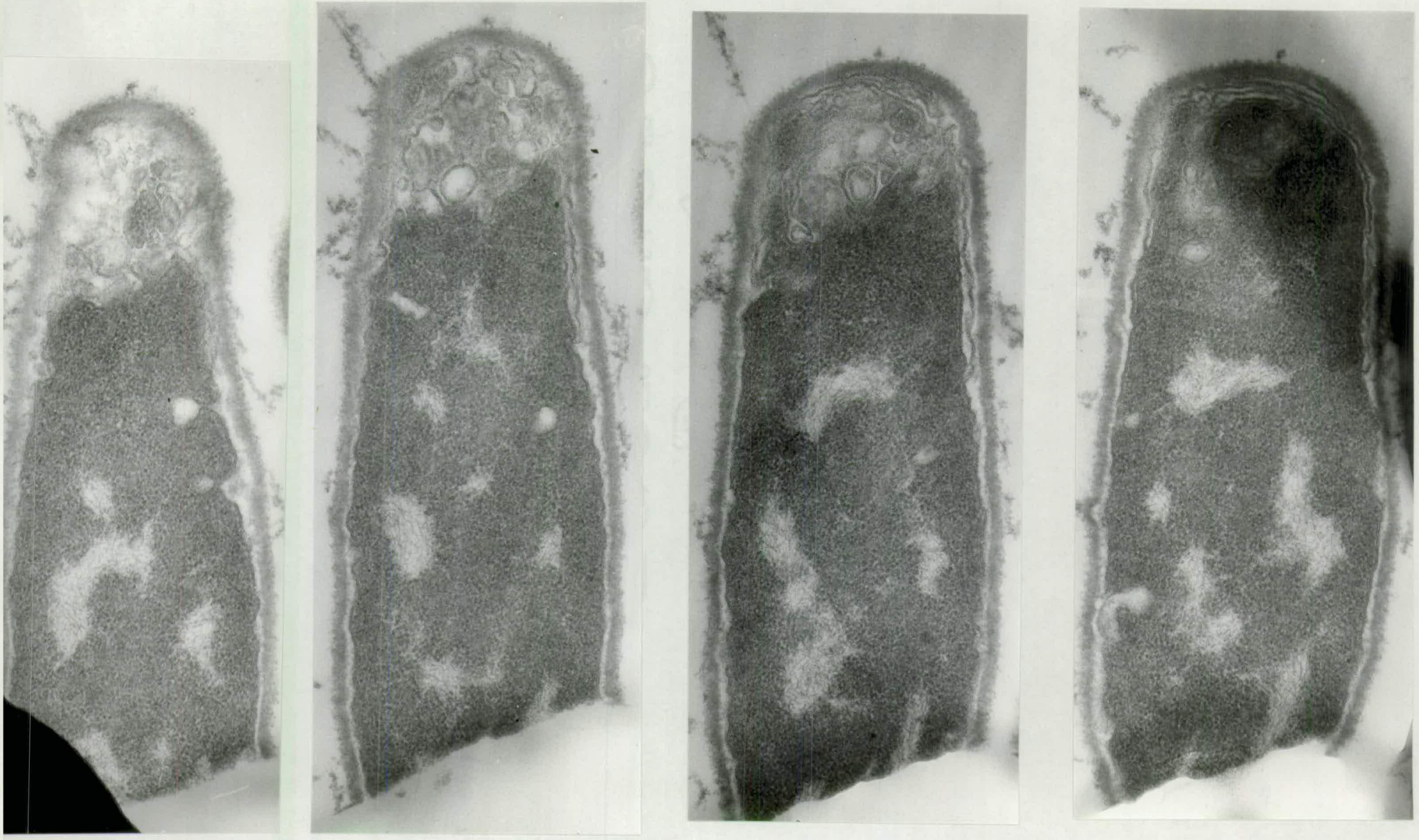
B. cereus 569. 30 min. treatment with A.D. Multiple layers of extra membrane. x 60,000

Plates 65 - 71

B. cereus 569, treated with A.D. 30' Serially sectioned cell. x 60,000







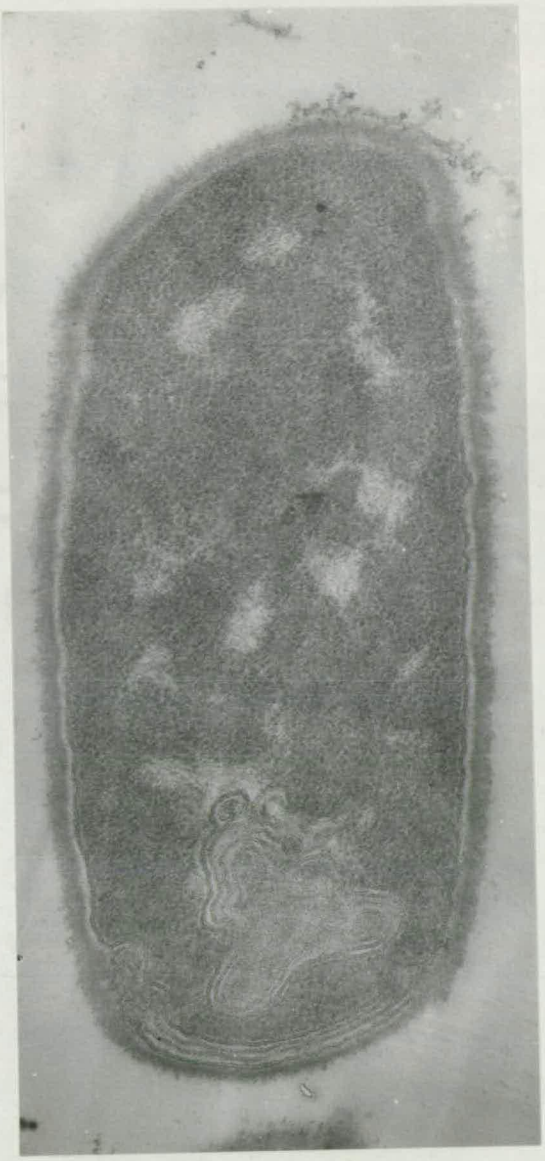


Plate 72

B. cereus 569. 30 min. treatment with A.D. at O.D. 0.74. Extra membranes. x 60,000

Plate 73

B. cereus 569. 30 min. treatment with A.D. at O.D. 0.74. Mesosomes with increased membrane content.

x 60,000

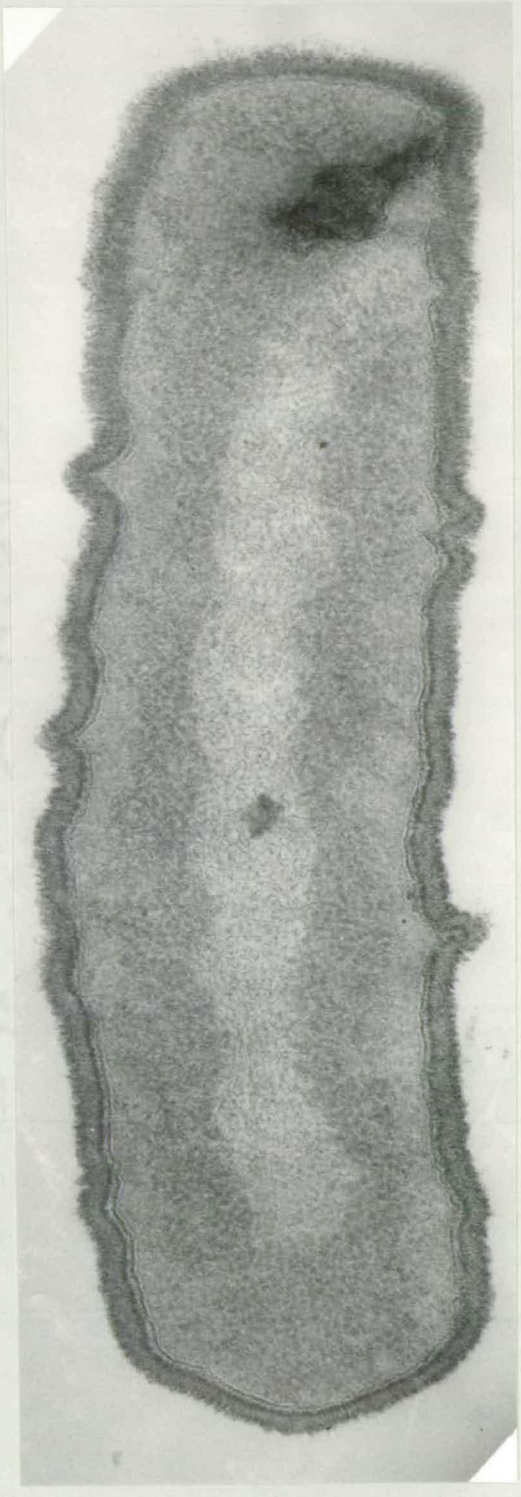


Plate 74

B. licheniformis 749c after 30 min. exposure to A.D.

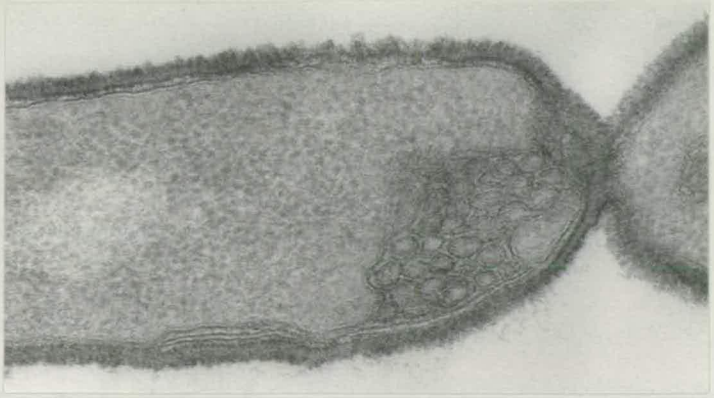


Plate 75

Disorganised mesosome with extra membrane in *B. licheniformis* 749c after A.D. treatment. 30' x 60,000

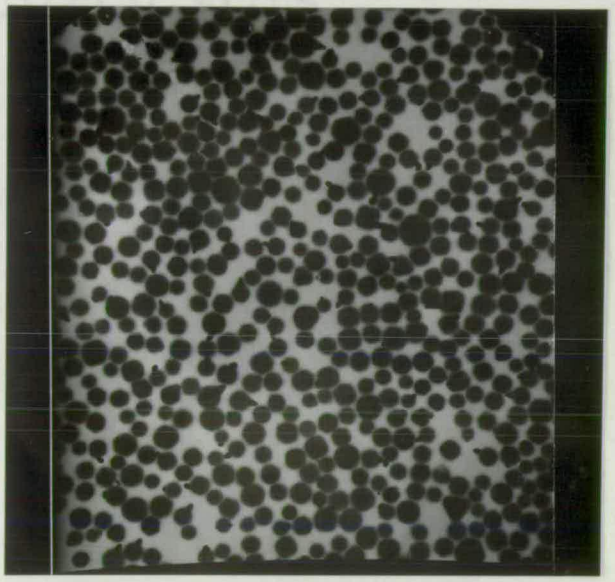


Plate 76

Silver monolayer, undeveloped. Contact print, x 20,000

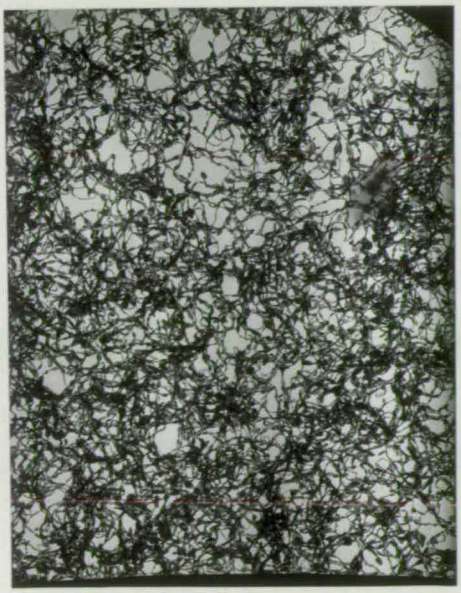


Plate 77

Silver monolayer, processed. Contact print, x 20,000

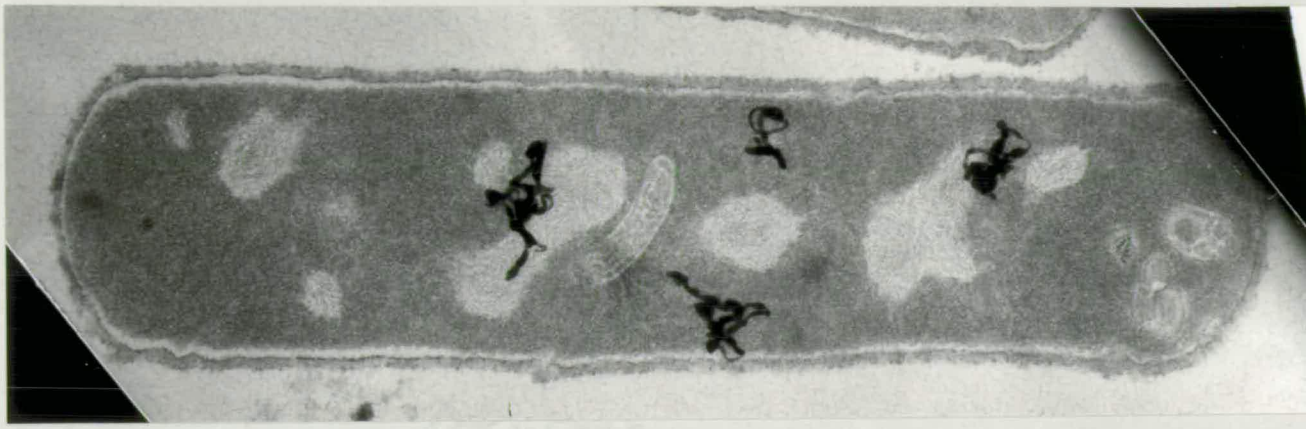


Plate 78

B. cereus 569. Control cell pulse-chased with G2T 15 min. x 40,000

Plate 79

Contact print of sample negative from autoradiography specimen. x 20,000

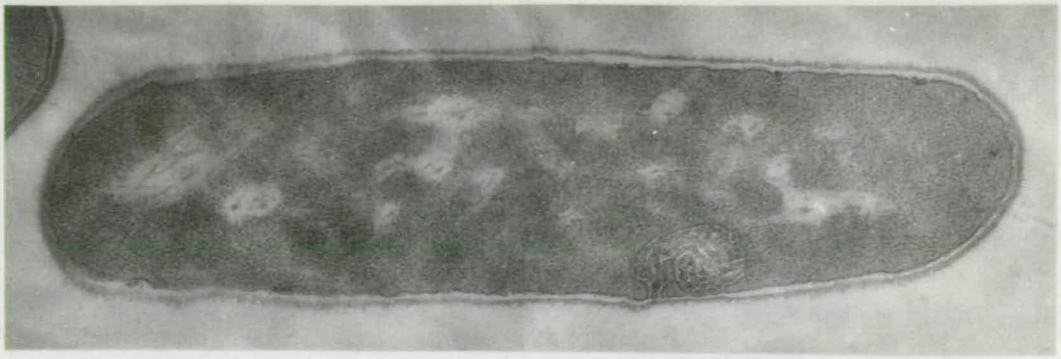


Plate 80

B. cereus control cells washed in buffer. x 40,000

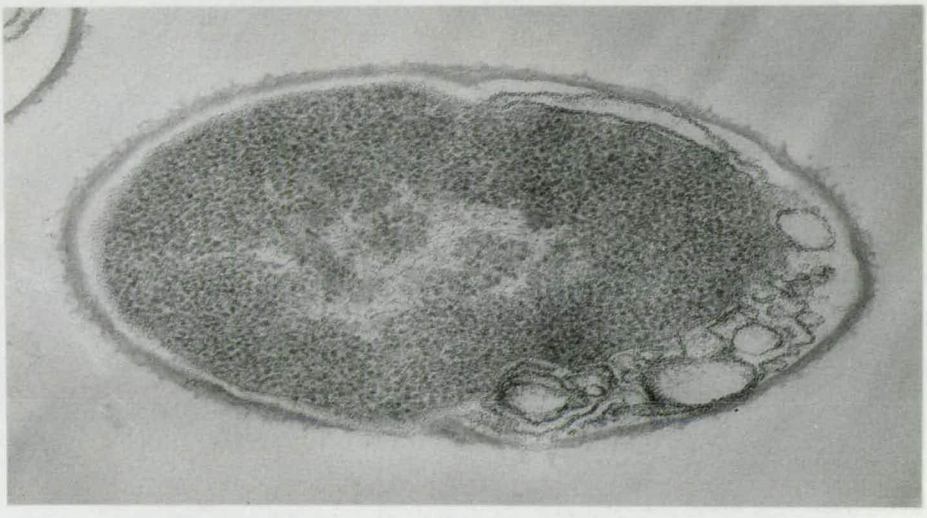


Plate 81

B. cereus. A.D. treated 30', washed in buffer. x 60,000

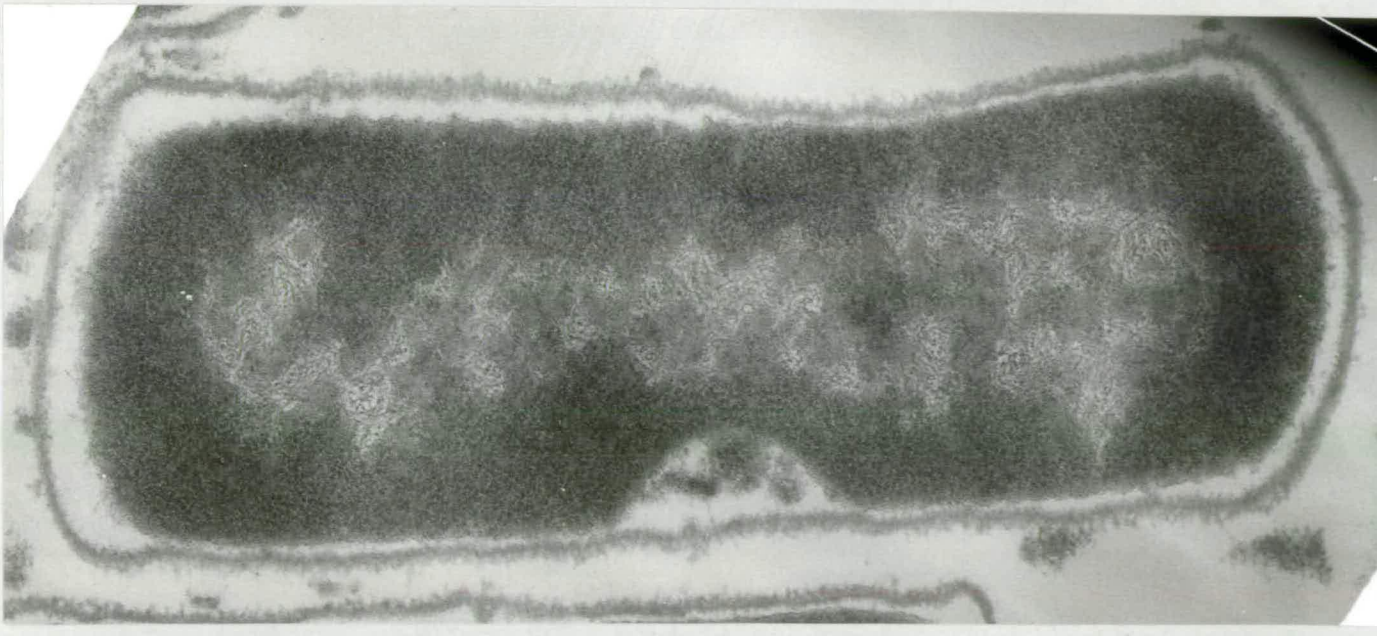


Plate 82

B. cereus 569. C-M extracted. x100,000

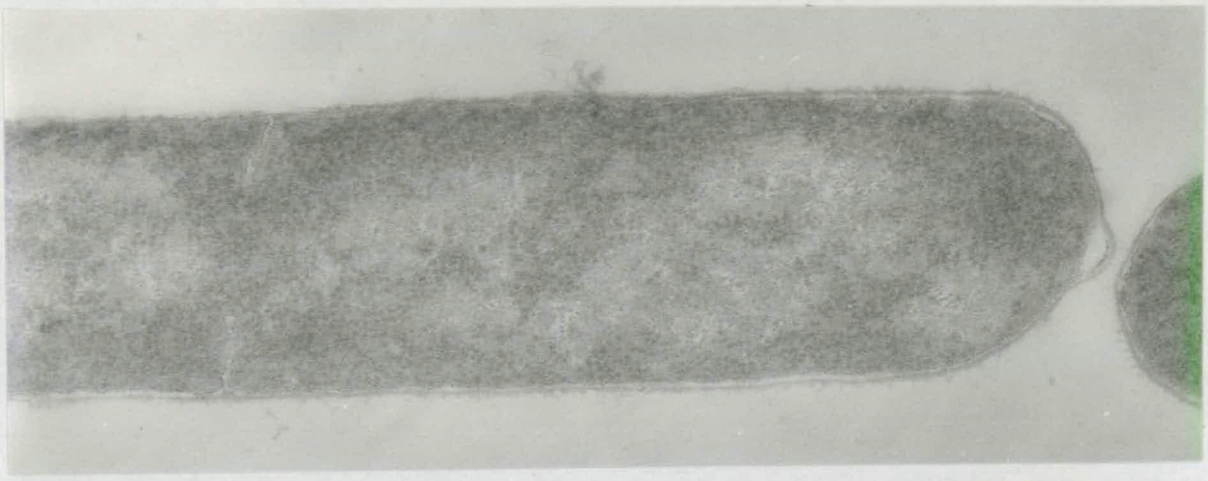


Plate 83

B. cereus 569 cells washed in sucrose buffer at 4°C.

×60,000

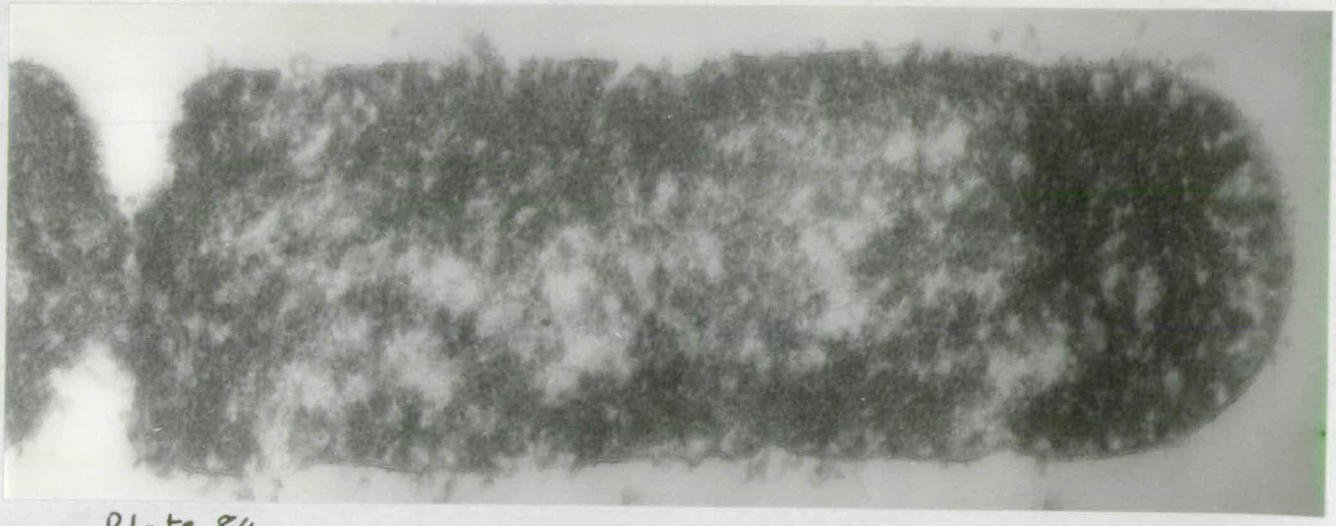


Plate 84

B. cereus. 30 min. lysosyme treatment in sucrose buffer. The cell shape is preserved but the cell wall has been removed.

×100,000